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GENETIC INTERACTIONS OF FACTORS THAT REGULATE ALTERNATIVE RNA SPLICING IN THE MALE GERM LINE OF DROSOPHILA

Shanzhi Wang

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by

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GENETIC INTERACTIONS OF FACTORS THAT REGULATE ALTERNATIVE RNA SPLICING IN THE MALE GERM LINE OF DROSOPHILA

A

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for the Degree of

DOCTOR OF PHILOSOPHY

by

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Houston, Texas

Dec 2012

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Abstract

Genetic interactions of factors that regulate alternative RNA splicing in the male germ line of Drosophila

Publication No._____

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Supervisory Professor: William Mattox, Ph.D.

Alternative RNA splicing is a critical process that contributes variety to protein functions, and further controls cell differentiation and normal development. Although it is known that most eukaryotic genes produce multiple transcripts in which splice site selection is regulated, how RNA binding proteins cooperate to activate and repress specific splice sites is still poorly understood. In addition how the regulation of alternative splicing affects germ cell development is also not well known. In this study, Drosophila Transformer 2 (Tra2) was used as a model to explore both the mechanism of its repressive function on its own pre-mRNA splicing, and the effect of the splicing regulation on spermatogenesis in testis. Half-pint (Hfp), a protein known as splicing activator, was identified in an S2 cell-based RNAi screen as a co-repressor that functions in combination with Tra2 in the

splicing repression of the M1 intron. Its repressive splicing function is found to be sequence specific and is dependent on both the weak 3’ splice site and an intronic splicing silencer within the M1 intron. In addition we found that in vivo, two forms of Hfp are expressed in a cell type specific manner. These alternative forms differ at their amino terminus affecting the presence of a region with four RS dipeptides. Using assays in Drosophila S2 cells, we determined that the alternative N terminal domain is necessary in repression. This difference is probably due to differential localization of the two isoforms in the nucleus and cytoplasm. Our in vivo studies show that both Hfp and Tra2 are required for normal spermatogenesis and cooperate in repression of M1 splicing in spermatocytes. But interestingly, Tra2 and Hfp antagonize each other’s function in regulating germline specific alternative splicing of Taf1 (TBP associated factor 1). Genetic and cytological studies showed that mutants of Hfp and Taf1 both cause similar defects in meiosis and spermatogenesis. These results suggest Hfp regulates normal spermatogenesis partially through the regulation of taf1 splicing. These observations indicate that Hfp regulates tra2 and taf1 activity and play an important role in germ cell differentiation of male flies.

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CHAPTER ONE

General Introduction

In the original idea of the central dogma (Crick, 1970; Crick, 1958), RNA was simply thought to be a middle carrier during a flow that transferred genetic information from DNA to protein. DNA encodes all the information necessary to make final protein products, while RNA will be used passively to copy that information from DNA and direct protein synthesis. Until 1977 (Berget et al., 1977) when RNA was discovered to be spliced before entering the cytoplasm to direct protein synthesis, people realized that there are many regulatory steps at the RNA level during gene expression.

1. pre-mRNA splicing

Eukaryotic genes contain both exons and introns. Exons are the regions that will be kept in final mRNAs but are split by non-coding introns. To make coding information intact for future protein synthesis, introns will be cut out and other parts will be connected together to form complete and useful information. This process is called pre-mRNA splicing.

Although split genes exist in eukaryotic cells, their prevalence in the genome is highly diverse across the species. In budding yeast, less than 5% of genes have introns and undergo RNA splicing. However this percentage can reach more than 90% in the human genome (Ares et al., 1999; Neuveglise et al., 2011; Pan et al., 2008; Shieh et al., 2011; Wang et al., 2008).

To get rid of introns from a linear nucleotide chain, splicing signals are required to distinguish introns from exons. In general

splicing signals are the short sequences residing the exon-intron boundaries and within the introns. The 5′ splice site occurs at a short conserved consensus sequence with GU as the most 5′ end of the intron. Near the opposite end of the intron there are two signals: an AG dinucleotide at the most 3' end that is preceded a polypyrimidine tract and a loosely conserved branch site sequence (Figure 1-1). Briefly, intron splicing takes place in two steps. First, the 2′-hydroxyl of an adenosine nucleotide at the branch point attacks the phosphate of the guanosine nucleotide at the 5′ splice site. This will release a free 5′ exon and a lariat product including the rest of the transcript. Second, the free 5′ exon will use its 3′-hydroxyl to attack the phosphate of 3′ splice site and ligate with the downstream exon, in the meantime the lariat intron is released (Figure 1-2).

RNAs have many important functions including catalysis. The “RNA world” hypothesis (Cech, 2011) suggests that RNA itself has the potential to function as an enzyme. Actually, the splicing reactions of some special introns could be catalyzed just by RNA itself (Bonen and Vogel, 2001; Nielsen and Johansen, 2009). However in terms of pre-mRNA splicing, proteins and protein-RNA complexes are required for RNA splicing to be accomplished correctly. These protein factors will recognize the splice sites, recruit other factors and form the catalytic complexes by communicating with each other to finally complete intron splicing (Wahl et al., 2009).

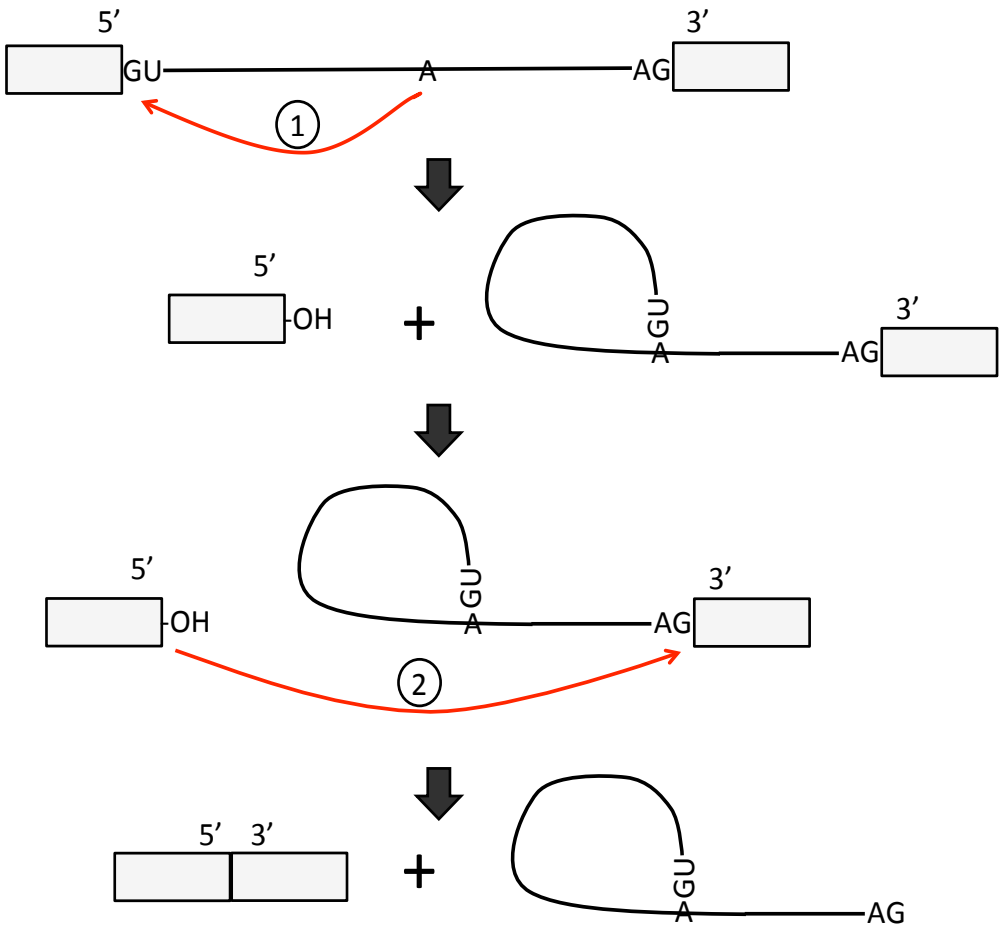


Figure 1-1. Two steps of catalytic reactions of pre-mRNA splicing

Gray boxes indicate exons and horizontal thin lines indicate introns. 5’ and 3’ represent 5’ splice site and 3’ splice site within the intron. GU are two starting nucleotides and AG are the ending nucleotides of the intron. “A” represents branch point site. Two catalytic steps are indicated as numbers. Reactions between residue groups are represented by red arrow lines.

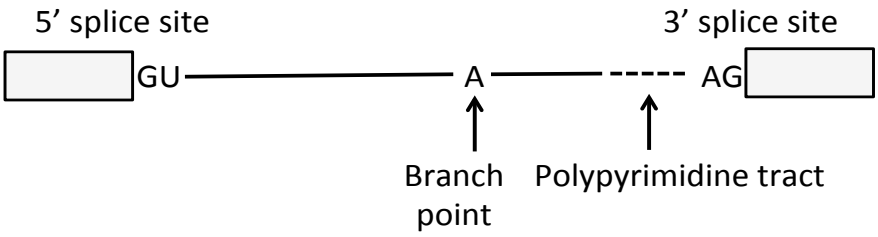


Figure 1-2. Basic splicing signals for pre-mRNA splicing

Exons are shown as gray boxes and intron is shown as the horizontal line. Polypyrimidine tract is shown as the dash line. GU/AG represent the nucleotides in the boundary between the exons and the intron.

It is known that the U1 small nuclear RNA particle (U1 snRNP) recognizes the 5′ splice site, splicing factor 1 (SF1) binds to the branch point, and U2 snRNP auxiliary factors (U2AFs) recognize the 3′ splice site and the polypyrimidine tract. At this point, the RNA-protein complex is called E complex. The U2 small nuclear RNA particle (U2 snRNP) is then recruited by interacting with U2AF, replacing SF1 and base-pairing with the branch point in an ATP-dependent manner to transform the E complex into A complex. Once a stable spliceosome is formed, tri-snRNPs (U4-U5-U6 snRNPs) will join in and transform A complex into B complex. After U1 and U4 snRNPs leave and the complex finishes rearrangement, the whole spliceosome becomes active and performs the catalytic function to complete splicing in two steps as discussed above (Black, 2003)(Figure 1-3).

With the genome becoming more complex, higher eukaryotes contain more multi-intron genes (Kim et al., 2008), and RNA splicing becomes more flexible to have many choices of determining which exons will be included in the final messenger RNAs. Thus this process eventually becomes alternative splicing instead of simple RNA splicing (or constitutive splicing). Alternative splicing patterns include alternative 5′ splice sites, alternative 3′ splice sites, exon skipping, the use of mutually exclusive exon and intron retention (Figure 1-4). Alternative splicing is thus a highly versatile mechanism for producing different mRNA isoforms from a single primary transcript.

Alternative RNA splicing is an essential process for human cells to produce various proteins that perform distinct functions

in tissue-specific or developmental stage-specific manners. As estimated by genome-wide studies, human cells have about 20,000 genes in their genome, but produce hundreds of thousands of distinct proteins. These proteins cannot be encoded in the genome by individual genes, so RNA alternative splicing provides a wonderful tool to expand the size of the protein pool and increase the functional diversity of the cells (Keren et al., 2010). The sequencing results with the new RNA-seq technique have shown that there are around 100,000 alternative splicing events in human cells (Pan et al., 2008). For an example, the Drosophila *Dscam* gene (Down syndrome cell adhesion molecule), which encodes axon guidance receptors, can produce 38016 mRNA isoforms by alternative splicing to meet its functional requirements in nervous system (Celotto and Graveley, 2001; Cooper et al., 2009; Olson et al., 2007). During development, alternative splicing plays operative roles in many cellular events, such as cell division, cell fate decision and tissue maturation (Cooper et al., 2009; Hammond and Wood, 2011).

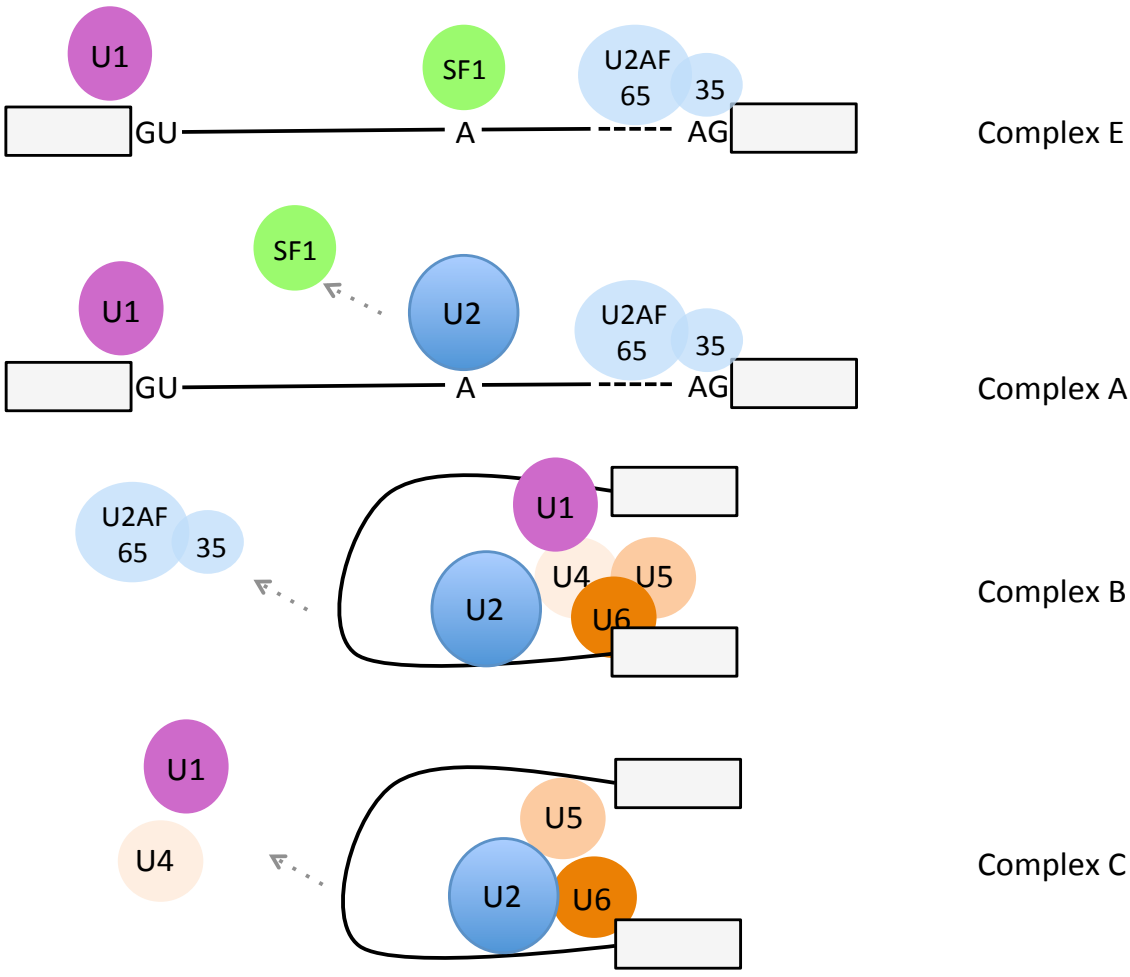

U1
SF1
U2AF
65
35
GU
A
AG
Complex E
SF1
U1
U2
U2AF
65
35
GU
A
AG
Complex A
U2AF
65
35
U1
U4
U5
U2
U6
Complex B
U1
U4
U5
U2
U6
Complex C

Figure 1-3. Dynamic changes of splicing complex

Exons are shown as gray boxes and introns are shown as horizontal lines. The transformations between the complexes are stated in detail in the text. GU and AG represent 5′ splice site and 3′ splice site respectively. “A” represents branch point site. U1, U2 and U4/U5/U6 represent small nuclear RNA particles. SF1 represents splicing factor 1. U2AF65 and U2AF35 are U2 snRNP auxillary factors 65 kDa and 35 kDa. Dashed arrows represent the splicing factors that leave from the complexes.

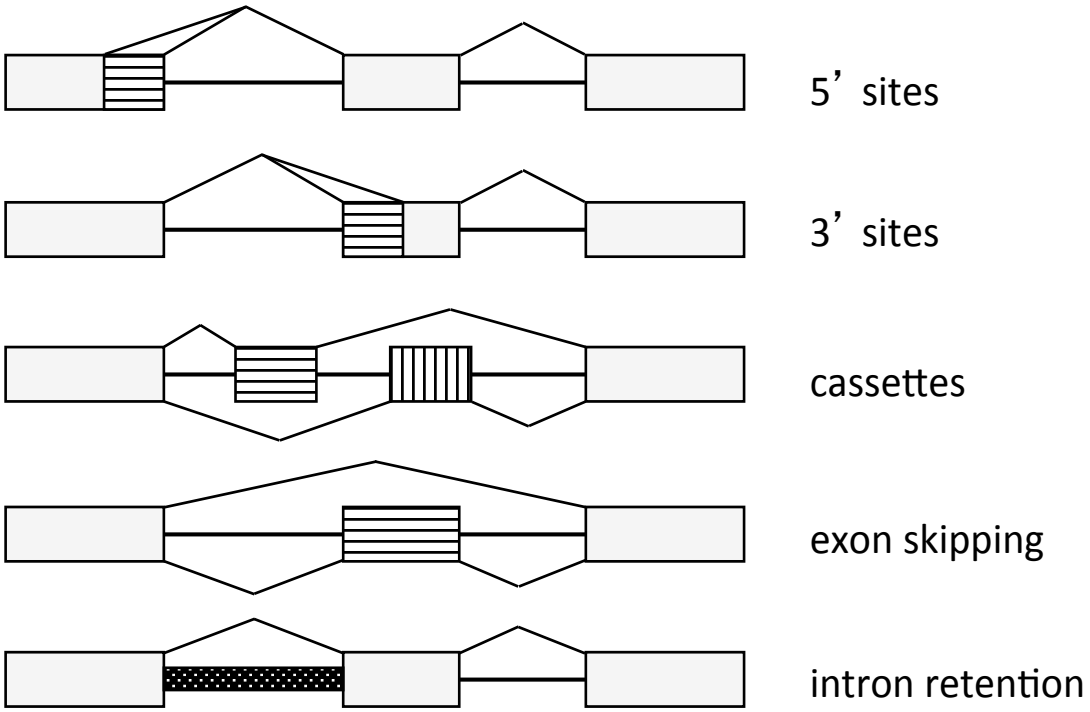


Figure 1-4. Alternative RNA splicing patterns

Diagrams showed different kinds of gene structures. Exons are shown as gray boxes and introns are shown as horizontal lines. Boxes with different shaded pattern represent alternatively selected exons. Splicing patterns are shown as the angled lines above and below the gene structures. Thickened line in the last diagram represents the retained intron.

However within complex genomes containing multi-intron genes, the limited consensus splice site sequences mentioned above cannot alone provide sufficient information to distinguish exons from introns. For instance, many exons with tissue specificity contain relatively weaker splice sites that are difficult for splicing factors to recognize (Berget, 1995). Also pseudo-splice sites within the introns will compete for splicing factors with those genuine ones (Zhang et al., 2005). Therefore the challenge for alternative splicing is to determine which splice sites should be used. Since it is critical to accurately define exons and remove introns, this process is undoubtedly highly regulated. Fortunately, other elements around splice sites are available and hundreds of regulators have been found to bind these elements to play either positive or negative roles that assist core factors of splicing machinery to make the right choices of splice sites.

2. Splicing regulators and cis elements

Besides core components of splicing complexes, hundreds of splicing regulators are required to make sure of the correct outcomes of alternative splicing. Generally, based on their roles on making decisions, splicing regulators could be regarded as either splicing activators or splicing repressors. If the factors function to promote recognition of certain splice sites, these factors are called splicing activators. On the contrary, if the regulators function to make splice sites less recognized, these factors are splicing repressors.

2.1 Splicing activators and positive regulation

Splicing activators are factors that can promote recognition of splice sites. Among the activators, SR (serine and arginine rich) proteins represent a well-studied activator family that functions most of the time to promote RNA splicing (Figure 1-5). They share some common features such as containing one RS (arginine and serine rich) domain in the C terminus, and one or two RRM (RNA recognition motif) domains in the N terminus. RS domain usually consists of RS-dipeptide repeats instead of randomly distributed arginine and serine. It is the target for post-translational modifications, and it also mediates protein-protein interactions (Wang et al., 1998; Xiao and Manley, 1997). The phosphorylation status will affect SR protein activities (Cao et al., 1997; Du et al., 1998), and also is responsible for protein shuttling between the nucleus and cytoplasm, contributing to their subcellular localization (Caceres et al., 1997). The RRM domains are mainly responsible for recognizing and binding RNA elements (Bourgeois et al., 2004), but in some cases, the RRM is also known to be involved in protein-protein interactions (Cho et al., 2011).

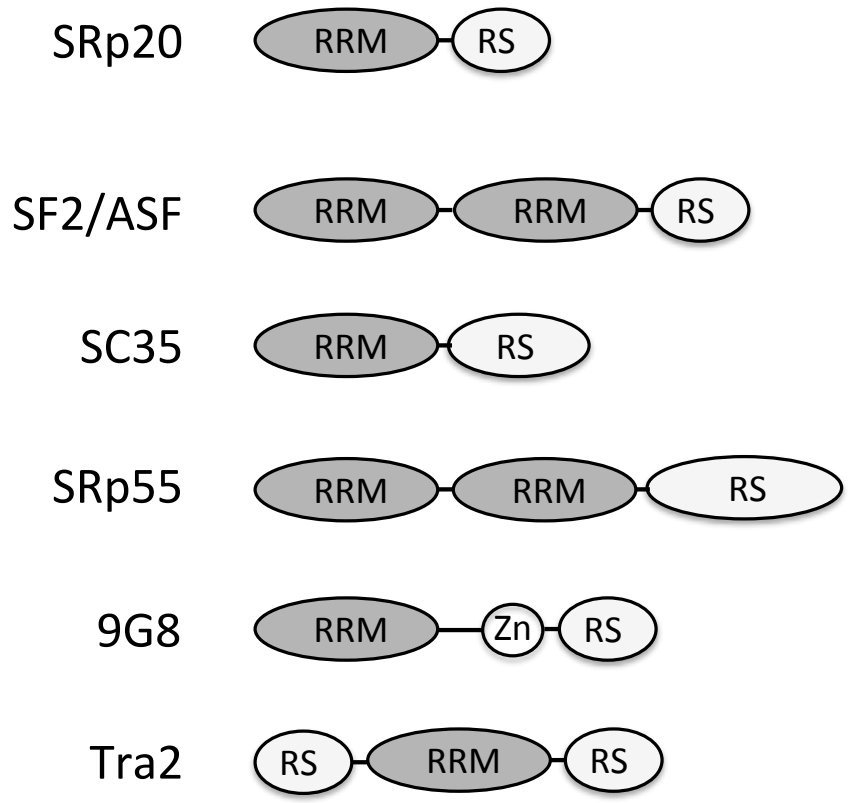


Figure 1-5. Domain structures of SR splicing factors

SR splicing family contains RS domains in their carboxyl terminus and one or two RRM in the amino terminus. RRM=RNA Recognition Motif, RS=arginine serine rich domain. Zn=zinc knuckle. Thin lines represent linking regions between the domains.

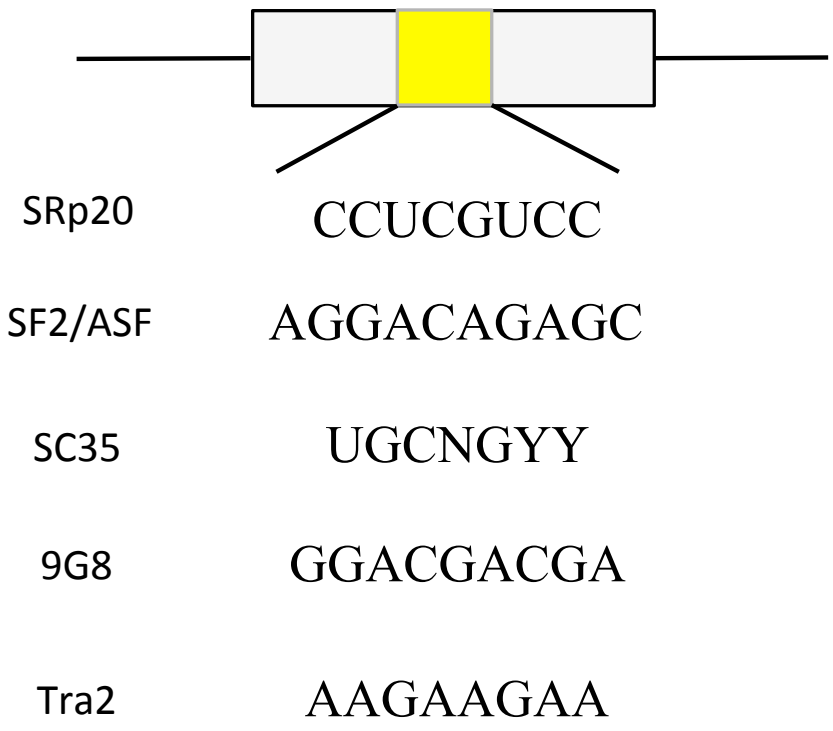


Figure 1-6. Sequences of exonic splicing enhancers

An exon is shown as the gray box. Thin lines represent the intron regions flanking the exon. The yellow box within the exon represents an exonic splicing enhancer. Individual recognition sequences of splicing factors are shown after the protein names.

The RNA elements bound by SR proteins are usually located within exons, called exonic splicing enhancer (ESE)(Figure 1-6). The enhancers recognized by SR proteins are either purine rich or pyrimidine rich (Cavaloc et al., 1999; Graveley, 2000; Schaal and Maniatis, 1999). Although different SR proteins each have individual binding site preferences, their recognition sites are highly degenerate (Liu et al., 1998; Sanford et al., 2009). This degeneracy of binding sites might contribute to the functional redundancy of SR proteins (Bourgeois et al., 2004; Graveley, 2000).

It has been found that the binding sites of SR proteins are highly enriched in exons compared with introns (Witten and Ule, 2011). It is believed that binding to the enhancer elements will help define the exon and facilitate preliminary spliceosome formation. A well-known example is from the alternative splicing of *doublesex* (*dsx*) pre-mRNA in Drosophila (Heinrichs et al., 1998; Inoue et al., 1992; Ryner and Baker, 1991)(Figure 1-7). The *dsx* gene is a key regulator for Drosophila's somatic sexual differentiation. In male flies, *dsx* pre-mRNA is spliced in a default way in which exon 3 is joined to exon 5 with exon 4 being skipped (Nagoshi et al., 1988; Salz, 2011). However in females, exon 4 is recognized due to the activity of complexes formed on its exonic splicing enhancer which contains six 13-nucleuotide repeats that bind Transformer (Tra) and Transformer 2 (Tra2) as well as Rbp1 and perhaps other SR proteins. Transformer (Tra) is a splicing factor expressed only in female flies, while the other factors including Tra2 and Rbp1 are non-sex-specific in the Drosophila soma. The formation of this complex on the ESE can promote

recognition of the weak 3′ splice site of exon 4 by facilitating the binding of the U2AF large subunit U2AF50 through interactions between the RS domains. Such interactions commit the intron to pre-spliceosome complex formation (Heinrichs and Baker, 1995; Inoue et al., 1992; Tian and Maniatis, 1993; Venables et al., 2012). Similar activity was also been observed in the regulation of the female specific 5′ splice site of another RNA substrate *fruitless* within the Drosophila nervous system, although the precise mechanism of activation has not been defined. (Heinrichs et al., 1998; Venables et al., 2012). Also studies of the dsx enhancers showed that the effect of each repeat on the splicing is additive instead of synergistic. This suggests that multiple SR-enhancer complexes increase the probability of a productive interaction between an enhancer complex and components of the spliceosome (Hertel and Maniatis, 1998).

The functions of RS domains are not limited to direct interactions with pre-spliceosomal proteins such as U2AF. Through secondary contacts with RNA sequence near the splice site itself, RS domains were also shown to stimulate RNA splicing in a distinct manner (Shen et al., 2004). When the RS domains were tethered to exon sequences, they were detected to also make contact with the branchpoint and promote spliceosome formation. The contact between the RS domain and the branchpoint was proposed to be relatively stable compared with the transient unstable interactions with other RNA sequences. This interaction is thought to facilitate the RNA base-paring between the U2 snRNA and pre-mRNA sequences at this site, or to prevent the association of inhibitory splicing factors

(Shen et al., 2004). The relative in vivo contributions to splicing activation of protein-protein interactions involving RS domains and those of RS-RNA interactions remains unclear.

Besides SR proteins, some other factors are also found to promote splicing by binding to exonic splicing enhancers. For example, YB-1, originally known as a DNA binding transcriptional factor, was shown to bind an AC-rich exonic enhancer to promote exon v4 recognition during the alternative splicing of CD44 in humans (Coulter et al., 1997; Stickeler et al., 2001).

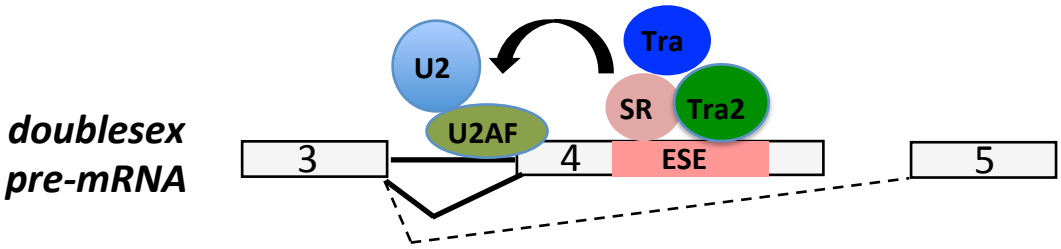


Figure 1-7. Alternative splicing of doublesex pre-mRNA

Splicing factors Transformer (Tra), Transformer 2 (Tra2) and another SR protein are assembled on the exonic splicing enhancer of exon 4, and help recruit U2AF and recognize the female specific weak 3′ splice site. The angled line represents the female specific splicing between exon 3 and exon 4. The dashed angled line represents the male-type splicing between exon 3 and exon 5.

2.2 Splicing repressors and negative regulation

Splicing repressors are those factors that can inhibit splice site recognition and consequently cause exon skipping or intron retention in the final mature mRNAs. These factors could repress splicing by binding to either exons or introns to interfere with early or late steps in the assembly of active splicing complexes. To exemplify typical mechanisms that are used by splicing factors to repress splicing I will describe several well-studied systems below.

First, splicing repressors can interfere with the primary recognition of splicing signals in pre-mRNAs. As discussed above, basic splicing signals include 5′ splice site, 3′ splice site, polypyrimidine tract and branch point. It was shown that hnRNP A/B could bind to an exonic splicing silencer of the vpr exon in HIV-1 pre-mRNA, nucleate more hnRNP A/B binding upstream of the silencer, and repress the exon splicing by competing the activity of U2AF65 in the upstream polypyrimidine tract (Domsic et al., 2003). Also in Drosophila, *Sex-lethal* (*Sxl*) represses the male specific exon splicing of *tra* pre-mRNA by binding the polypyrimidine tract of the male-specific 3' splice site and blocking the essential splicing factor U2AF65 in this site (Valcarcel et al., 1993)(Figure 1-8A).

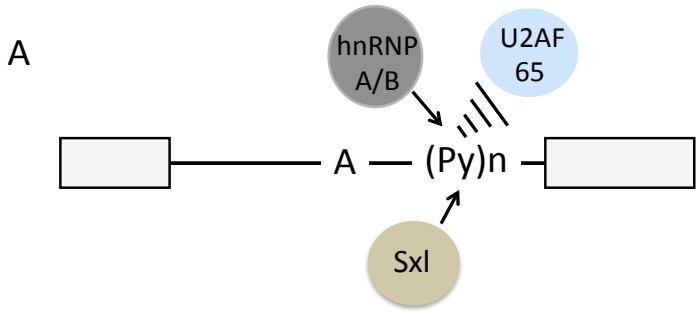
Other splicing repressors are known to compete and antagonize the functions of splicing activators. For example, In HIV I tat exon 2, hnRNP A1 was shown to bind in the exonic spicing silencer which is overlapping with another exonic splicing enhancer recognized by the splicing activator SC35. It is thought that hnRNP A1 represses tat exon 2 inclusion by directly competing out SC35′s

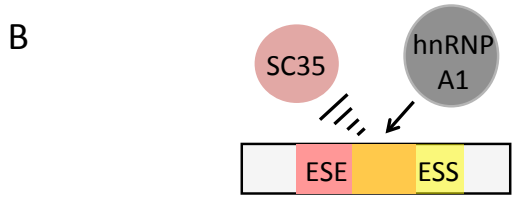
activity within the exon. Mutation of the hnRNP A1 binding site in the ESS will bring back the efficient splicing that is promoted by the binding of SC35 with high affinity (Zahler, 2003)(Figure 1-8B).

In some cases splicing repressors could form a loop structure in the pre-mRNA and exclude the regulated exon from the active splicing complex. This kind of regulatory behavior is observed in several hnRNP family members. Splicing factors bind the sequences in both the upstream and downstream introns flanking the regulated exon. Then the proteins will interact with each other, loop out the middle exon, and cause the exon skipping. In this way hnRNP A1 promotes exon skipping in its own pre-mRNA (Black, 2003; Blanchette and Chabot, 1999; Rooke et al., 2003). Similar mechanism has been suggested to be responsible for the splicing of the c-src N1 exon regulated by PTB and hnRNP F/H complex in neuronal cells (Rooke et al., 2003)(Figure 1-8C).

Still another proposed negative mechanism involves the interference with specific steps in active spliceosome assembly. During spliceosome assembly, new factors will join the complex and some factors will leave at the same time. The spliceosome will undergo a number of conformation changes between E complex and B complex until it becomes catalytically active. Some splicing repressors act by disrupting the intermediates in this process resulting in the formation of inactive complexes that cannot progress to a catalytically active state. For example, RBM5 was reported to stall the spliceosome transition after complex A formation by blocking the incorporation of U4/U5/U6 tri-snRNP into complex A formed on intron 5 and intron 6. In this way, RBM5

promotes exon 6 skipping in Fas substrate (Bonnal et al., 2008)(Figure 1-8D).


A
hnRNP
A/B
U2AF
65
A
(Py)n
Sxl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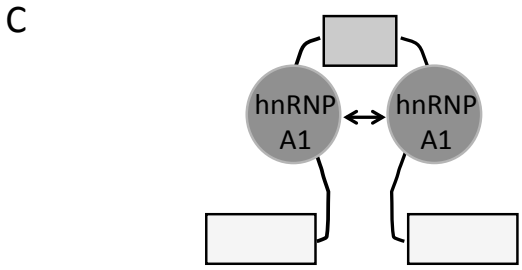
SC35
hnRNP
A1
ESE
ESS


C
hnRNP
A1
hnRNP
A1

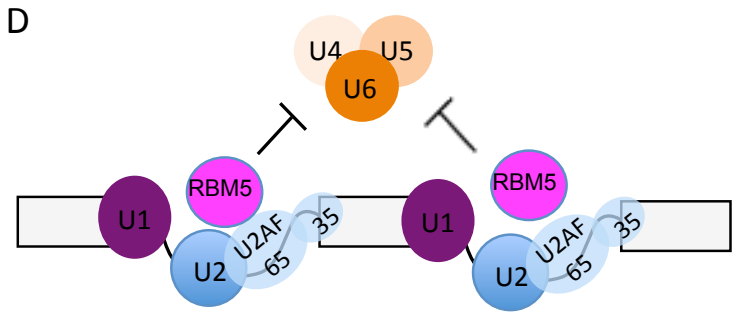

D
U4
U5
U6
RBM5
U1
U2
U2AF
65
35
RBM5
U1
U2
U2AF
65
35

Figure 1-8. Models of splicing repression

(A) hnRNP A/B competes with U2AF65 to bind the polypyrimidine tract within the 3′ region of the intron, causing 3′ splice site less recognized. Sex lethal (Sxl) competes with U2AF65 to associate with the 3′ region and prevent more mature splicing complexes from forming. (B) The exonic splicing silencer (ESS) overlappes with an exonic splicing enhancer. Binding of hnRNP A1 to the ESS will compete with SC35, which associates with the same site within the exon. (C) hnRNP A1 binds both upstream and downstream of the regulated exon. The self-interactions between hnRNP A1 molecules loop out the middle exon and cause its splicing repression. (D) RBM5 prevents the U4/U5/U6 tri-snRNPs from entering complex A formed in intron 5 and intron 6, causing the exon 6 skipping.

3. Position dependent effects of splicing regulators

When the hnRNP family factors such as hnRNP A/B, hnRNP A1 and PTB were first found to regulate alternative splicing, they were identified as splicing repressors (Caputi et al., 1999; Mayeda and Krainer, 1992; Singh et al., 1995). Follow-up studies also proved time and time again that these factors are mostly involved in the splicing repression (Han et al., 2010; Spellman and Smith, 2006). SR proteins were initially identified as splicing activators (Zahler et al., 1992). And their positive roles on alternative splicing are also been verified repeatedly in extensive studies on their mechanisms of actions (Bourgeois et al., 2004; Graveley, 2000). Therefore there is an impression that hnRNP factors can generally be regarded as splicing repressors while SR protein family should be an important class of splicing activators.

However, these observations are not universal and a number of exceptions to this general categorization have come to light. In some cases it has been found that hnRNP family proteins can promote splice site recognition, while SR proteins also have the ability of directly repressing the recognition and utilization of specific splice sites.

One example of this is provided by the hnRNP H protein which is known to be a component of splicing enhancer complex binding downstream of c-src alternative exon N1. Activation by hnRNP H occurs when it forms a heterodimer with hnRNP F to promote the N1 inclusion in neurons by cooperating with SR proteins and a neural specific PTB within an enhancer complex (Chou et al., 1999). An in vitro study with a larger intron also showed that hnRNP A can

stimulate intron splicing with the cooperation of hnRNP H by promoting an intron definition (Martinez-Contreras et al., 2006).

These exceptional cases could be restricted to particular substrates or interacting factors. However, is it possible that a general principle hiding behind these observations could explain the positive or negative functions performed by the same family of splicing regulators? With more and more exceptions discovered, the protein binding location is emerging as an important factor in determining whether the activation or repression of splicing is observed from a given regulator.

With the development of the technique of RNA-seq, genome-wide studies on the alternative splicing by individual regulators has become possible. Several important splicing factors in nervous system development have been studied in humans and their binding targets have been explored at the transcriptome scale (Jensen and Darnell, 2008; Ule et al., 2003). In such studies all alternative splicing events were identified, and the effects of mutations in splicing regulators on the alternative splicing were compared. By combining transcriptome sequence with in vivo RNA-protein crosslinking and immunoprecipitation it was found that when Fox1/2 bound the downstream intron of an alternatively spliced exon, it could promote the exon's inclusion, while binding in the upstream intron will cause the exon's skipping. The protein location determines its role during the RNA splicing (Zhang et al., 2008). Similar effects were also observed in other splicing factors like NOVA and some hnRNP family members (Dredge and Darnell, 2003; Ule et al., 2006)(Figure 1-9).

Models have been proposed to explain how the binding locations may be responsible for different splicing consequences (Witten and Ule, 2011). When RBPs (RNA binding proteins) bind upstream of the alternatively spliced exon, they interfere with the recognition of splice signals in the upstream intron and cause the exon's skipping (Chen and Manley, 2009). Fox-1 was shown to repress the splicing of exon 4 of the calcitonin/CGRP pre-mRNA by binding a UIF (upstream intronic flanking) region, blocking SF1 from binding the branch point nearby, and finally preventing E complex formation (Zhou and Lou, 2008). When binding to the downstream intron, RBPs help to bridge splice sites and promote splicing (Chen and Manley, 2009; Licatalosi and Darnell, 2010). In a study of the alternative splicing of a cassette exon N30 in NMHC (nonmuscle myosin heavy chain) II-B pre-mRNA, another Fox family factor, Fox-3, was found to activate N30 inclusion through binding an element within the downstream intron (Kim et al., 2011). More interestingly, the active function of Fox is dependent on another splicing factor PSF (protein-associated splicing factor) in this substrate (Kim et al., 2011). This suggests that the position-dependent regulation of splicing by Fox protein is involved with another layer of regulation and might also be determined by the interactions between splicing regulators. In some cases, Fox and Nova could regulate the same substrate synergistically (Zhang et al., 2010). Fox2 was found to bind the 5′ end of intron 9 from Gabrg2 pre-mRNA, while Nova bound to the region 3′ of it. It was supposed that by interacting with each other, Fox2 and Nova looped out intron 9 and promoted exon 9 splicing in humans (Zhang et al., 2010). Even though more

details of these mechanisms need to be clarified, these studies have clearly shown the importance of the binding locations of RNA binding proteins in terms of the regulation of alternative splicing.

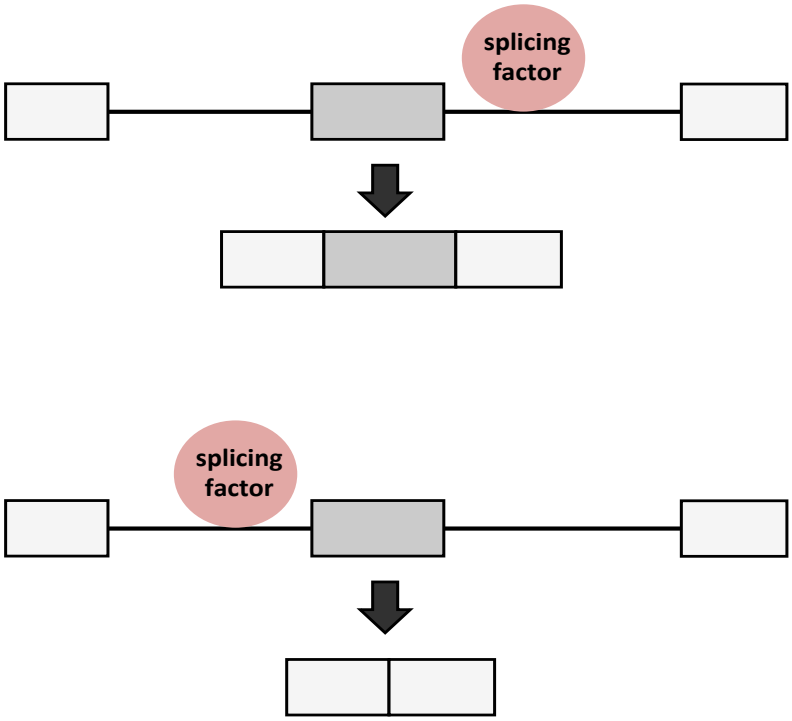


Figure 1-9. Position dependence of splicing regulation

Gene structure is shown with gray boxes and horizontal lines, which represent the exons and introns respectively. Middle darker box represents a regulated exon. When splicing factors bind downstream of the alternatively spliced exon, they will help the exon inclusion. While binding upstream of the exon, they will favor the exon's skipping.

4. Splicing repression by SR proteins

Studies on several pre-mRNAs have revealed the roles for SR splicing factors in the negative regulation of alternative splicing. An example is from studies on the role of SF2/ASF in the splicing of adenovirus IIIa transcript (Figure 1-10). In the early stage of infection, SF2 could bind the element within the intron of the exon IIIa and represses the recognition of the distal 3′ splice site, resulting in the inclusion of a longer exon in early viral mRNA (Kanopka et al., 1996; Simard and Chabot, 2002). Another case is related with SRp30c, which is shown to bind an intronic splicing silencer CE9 of intron 7B in hnRNP A1 pre-mRNA, which causes less recognition of the downstream 3′ splice site, and finally in the skipping of exon 7B (Simard and Chabot, 2002). In CFTR substrate, the SR proteins SRp40 and SF2/ASF form a complex on an intronic splicing silencer in intron 9, repress the recognition of upstream exon 9 and cause its skipping (Buratti et al., 2007) (Figure 1-14). All of these cases share a common feature that SR proteins repress RNA splicing by binding to the intronic sequences.

How do SR proteins repress the RNA splicing? Some SR proteins appear to have intrinsic activities that results in the general repression of splicing. SRp38, a mammalian specific SR protein, can repress pre-mRNA splicing in a cell cycle dependent way (Shin and Manley, 2002). Its effect on splicing can be switched from repressive into active by its phosphorylation status (Feng et al., 2008). However SRp38 differs from other SR proteins in that it does not act as a general splicing activator. Even in S100 extract, a cell free system which is absent of SR proteins but with most other

splicing components, SRp38 did not activate splicing as other SR proteins did (Feng et al., 2008). Another SR protein SRrp86 was shown to repress splicing by interacting and interfering with the active functions of other SR proteins such as SC35, ASF/SF2 and SRp55 (Barnard et al., 2002).

Some biochemical properties of these factors also provide clues. Unlike other SR proteins, SRp38 contains two RS domains in the C terminus. Both of these RS domains perform repressive activities. However the repression by RS1 alone is dephosphorylation-dependent and this domain has only modest activation function. The second RS domain RS2, containing some SK dipeptides, displayed a novel second-step repression but without dephosphorylation-dependent repression and activation ability (Shin et al., 2005). The SRp38 RNA binding domain (RBD) shows more similarity with the RRMs of U2AF homology motif family (UHM) rather than the canonical RNA binding motifs in other SR proteins. This UHM-like RBD has no activation function, but can prevent the RS1 domain from functioning with positive activity. SRrp86 also contain an EK-rich domain which is believed to be responsible for its negative activity in RNA splicing (Li et al., 2002). SF2/ASF contains two RRMs at its N terminus, as do several other SR factors. The second RRM is atypical in sequence and was shown to be critical for its repressive activity in the splicing of Adenovirus IIIa (Dauksaite and Akusjarvi, 2002). This function seems to depend on a conserved “SWQDLKD” motif in the second RRM which was shown in tethering experiments to perform an effector function necessary for splicing repression (Dauksaite and Akusjarvi, 2002).

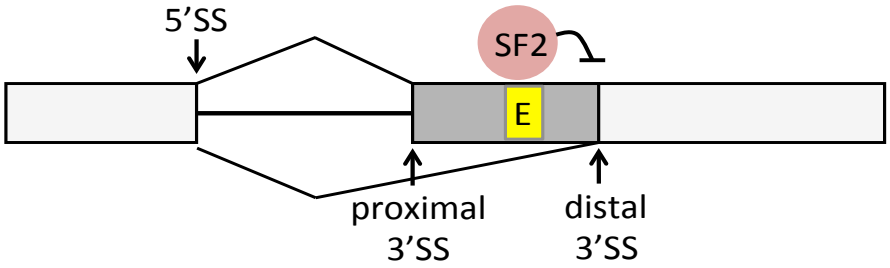


Figure 1-10. ASF/SF2 represses the distal 3′ splice site recognition

The adenovirus pre-mRNA structure is shown. The angled lines represent the splicing patterns. Without SF2, the distal 3′ splice site will be recognized. When SF2 is present, it will associate with the splicing silencer in the intron and repress the recognition of the distal 3′ splice site. E represents the intronic element.

4.1 Negative auto-regulation of transformer 2 splicing

Transformer 2 is a Drosophila SR-related protein. As I discussed above, it is well-known for its positive regulatory function in *doublesex* (*dsx*) alternative splicing where it plays an essential role for the formation of splicing enhancer complexes and for promoting recognition of a weak female specific 3′ splice site.

Besides its positive function, Tra2 was found to be responsible for the retention of the M1 intron in Tra2 transcripts produced in growing spermatocytes (Mattox and Baker, 1991; Mattox et al., 1996; Mattox et al., 1990). The M1 intron is alternatively spliced in the male germline, and the resulting mRNAs encode different Tra2 protein isoforms (Figure 1-11). The mature mRNA without the M1 intron will use the start codon located at the junction of exon 3 and exon 4, therefore it will be translated into a 226-amino acid peptides called Tra2-PC. In mRNA where the M1 intron is retained this start codon is split and a downstream start codon within exon 4 has to be used to produce a 179-amino acid peptides called Tra2-PE (Mattox et al., 1996). In wild type germ cells about 50% of mature mRNAs retain the M1 intron, but in the absence of Tra2 function, the intron is efficiently removed from germline transcripts and the M1 retaining transcripts were not detected. Other introns in the RNA are unaffected by the presence or absence of Tra2 function (Mattox and Baker, 1991). Genetic studies have shown that Tra2-PC is both required and sufficient for M1 retention in the germline (Mattox et al., 1996). Experiments in which negative auto-regulation was blocked by the deletion of the M1 intron showed that this mechanism served to prevent high-level

expression of Tra2 that would block normal spermatogenesis (McGuffin et al., 1998).

In vitro studies have identified several cis-acting elements involved in the M1 splicing (Chandler et al., 2003; Qi et al., 2007). An intronic splicing silencer (ISS) located upstream of the branch point was shown to bind Tra2 and was able to mediate the repression of intron splicing in vitro when it was inserted into the otherwise unregulated ftz intron (Figure 1-12).

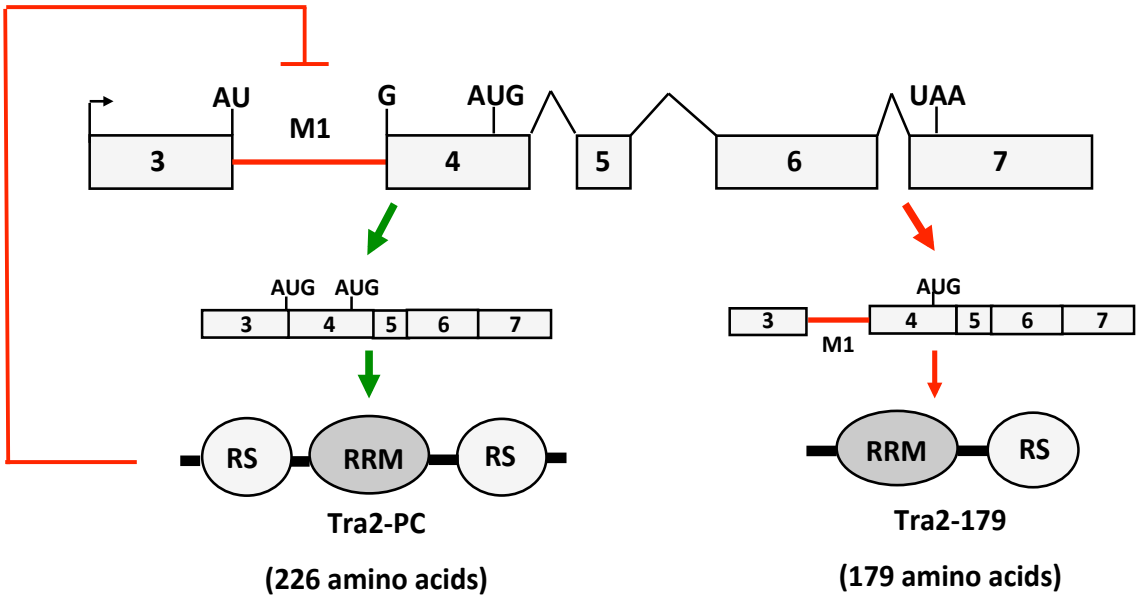


Figure 1-11. Alternative splicing of transformer 2 pre-mRNA

Transformer 2 gene structure is shown. The gray boxes represent the exons and the horizontal line represents the M1 intron. The transcriptional start site is indicated. Two start codons and the stop codon are labeled above the exons. The first start codon is split by M1 intron. Protein products from two mRNA isoforms are shown at the bottom. The mRNA using the first start codon encodes Tra2-PC containing 226 amino acids, while the M1 retained mRNA encodes Tra2-PE with 179 amino acids. Tra2-PC is able to repress M1 intron splicing in an auto-regulated manner.

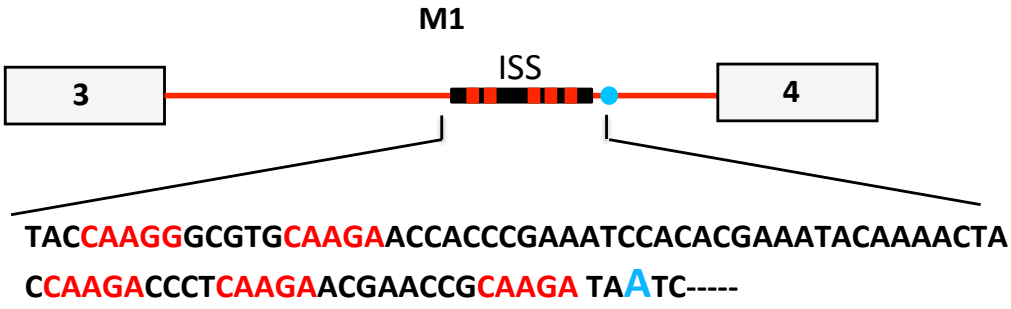


Figure 1-12. The intronic splicing silencer of M1 intron

A section of the tra2 pre-mRNA is shown. The horizontal line represents the whole M1 intron. The Black bar represents the intronic splicing silencer. Red bars represent the binding repeats of Tra2 protein within the ISS. The whole ISS sequence is shown below the diagram. Red letters are the CAAGR (R=A/G) repeats recognized by Tra2. The blue "A" is the predicted branch point site.

4.2 Position-dependent splicing repression by SR proteins?

As I discussed in section 3, it is found that in a genome-wide scale, hnRNP family and other RNA binding proteins exhibit position-dependent effects on regulating alternative splicing.

A similar situation has also been observed for SR proteins. Taking the adenovirus IIIa pre-mRNA as an example (Kanopka et al., 1996), when the 3RE element bound by SF2/ASF within the upstream intron was moved into the downstream exon, it could be used as an splicing enhancer to promote exon splicing by the same SR protein ASF/SF2. The ISS element in CFTR intron 9 was also found to exert an exonic enhancer function when it was moved to an exon (Buratti et al., 2007). A systematic study of this issue is found in work on Drosophila transformer 2 (Qi et al., 2007). The ISS element was found to contain five CAAGR repeats that bind Tra2 protein and are necessary for splicing repression to occur. These repeats are located upstream of the branch point but also mediated repression from distant intronic positions. This suggests that Tra2-ISS complex does not act to simply block the splicing signals, but instead represses through other interactions. Interestingly Tra2/ISS interactions could exhibit splicing enhancer activity when the ISS was inserted into the dsx exon (Shen and Mattox, 2012). Further, using an MS2 protein tethering assay (Shen and Mattox, 2012), Tra2 was found to perform a similar repressive function when it was tethered at different positions within the ftz intron, but activated splicing when tethered in dsx exon 4 or an exon in a ftz RNA substrate. These observations also suggest that the opposite

effects were not due to the differences between the ISS and ESE but rather due to the binding positions of the protein (Figure 1-13).

Why does the binding position matter? Why do SR proteins prefer to repress splicing when they bind within the introns but promote splicing when they bind in exons? So far no example has been observed where SR proteins can repress splicing through binding to an exon. One explanation depends on "exon definition". Considering the ability of SR proteins to promote splice site recognition and to recruit U2AF and U1 snRNP to the 3′ and 5′ splice sites, SR proteins could be regarded as “exon definers”. That is to say that a range of sequences bound by SR proteins will be defined as belonging to the exon. More intriguingly, if previously defined intron sequences are bound by SR proteins, this SR-bound intron part will be “exonized”.

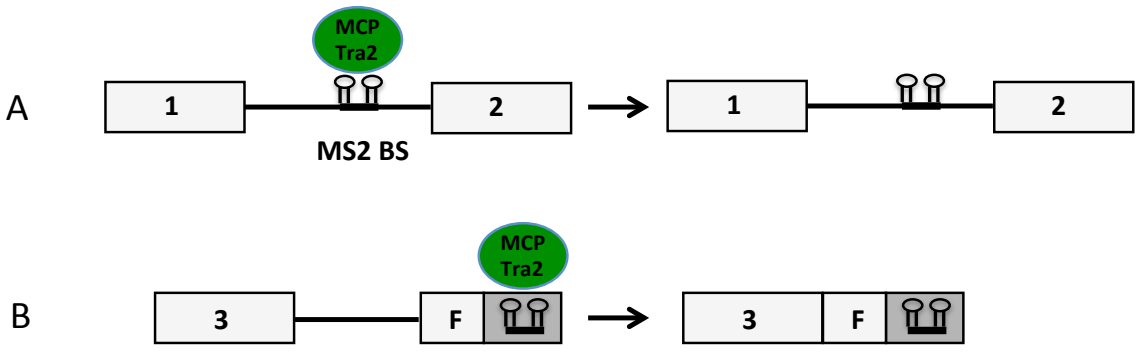


Figure 1-13. Position dependent regulation of RNA splicing by Tra2

(A) When Tra2 is tethered within the ftz intron, it will cause the splicing repression and the intron is retained in the final mRNA product. (B) When Tra2 is tethered in the female specific exon of dsx, it will promote the exon splicing with no intron remained in the final mRNAs. MCP-Tra2 is the fusion Tra2 protein with the amino acids from MS2 coat protein in the N terminus. MS2 BS is the MS2 binding sites shown as stem loops.

However, this does not explain how “exonizing” of an intron would cause splicing repression. For instance, in the case of hnRNP A1 pre-mRNA, SRp30c was known to bind the intron upstream of exon 8 but caused exon 8 skipping instead of inclusion of a longer exon inclusion (Simard and Chabot, 2002). Another important factor for consideration is the availability of a splice site nearby. Exon definition can only make a preliminary decision that the parts SR protein bound could be a potential exon. But to complete exon definition, the availability of nearby splice sites outside the bound region is also necessary. This idea is supported by the experiments with CFTR RNAs (Buratti et al., 2007)(Figure 1-14). When the people created a novel 5′ splice site downstream of an ISS within the intron 9, the resulting spliced products include a new exon using a pseudo 3′ splice cite just upstream of the ISS. Interestingly the alternative splicing of adenovirus regulated by SF2/ASF also suggested a similar mechanism in which the intronic part containing the ISS was exonized. And there was a 3′ splice site available just upstream of the SR binding sites, consequently the final mRNA contained a longer terminal exon 5265K (Kanopka et al., 1996)(Figure 1-10). If no splice site is available around the SR binding sites, the exon will be skipped or the intron will be retained. When Tra2 binds to the ISS element in the M1 intron, it helps define the intron part as an exon. However in this case no 3′ splice site is present upstream of the ISS within M1, and so the direct consequence is the retention of the intron.

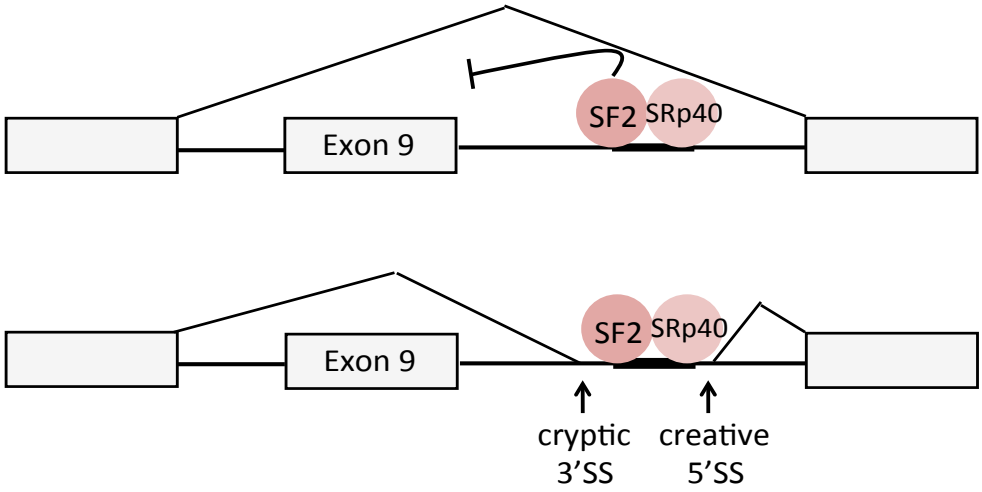


Figure 1-14. Splicing regulation of CFTR pre-mRNA

The exons and introns of partial CFTR pre-mRNA are shown as gray boxes and horizontal lines. The angled lines represent splicing patterns. When SF2 and SRp40 bind to the intron 9, they repressed exon 9 splicing. However when a new 5' splice sites was created around their binding sites, a new exon will be defined and included by using an upstream cryptic 3' splice site.

To further test the idea, it would be interesting to see how the Tra2-induced splicing pattern will change when a 3′ splice site is created upstream of the ISS within the M1 intron. A logical prediction is that a smaller intron upstream of the ISS would be actively spliced in a manner stimulated by Tra2. Exactly this kind of splicing event occurs in Tra2 transcripts from *Drosophila virilis* (Chandler et al., 1997), another Drosophila species that is about 60 million years diverged from *Drosophila melanogaster*. Within the vM1 intron (Drosophila virilis M1 intron), as shown in figure 1-15, there are several scattered CAAG repeats similar with the Tra2 binding sites in M1 intron. A 3′ splice site in this case is available just upstream of the CAAG repeats. Consequently vM1 intron retention could be achieved incompletely with a short fragment of vM1 intron being cut off (type B mRNA in figure 1-15). Another similar phenomenon is also seen in the splicing regulation of a testis-specific exon TLE4 by human Tra2. As shown in figure 1-16, hnRNP G forms a protein complex with other factors, and help recognize the exon T with the weak 5′ splice site but a strong 3′ splice site. When the Tra2 level is elevated, it binds to the repeated sites upstream of the exon T to form a larger complex. Another weak 3′ splice site is located just upstream of the Tra2 binding sites, resulting in the definition of a longer exon B in the presence of Tra2 (Liu et al., 2009).

Although this model could explain some repressive phenomena mediated by SR proteins, we still need to keep in mind that the final spliced mRNAs are the products of both positive and negative regulations. Even though we saw the splicing repression by SR

proteins, it is still possible that other splicing repressors could bind to other elements and antagonize the positive functions from SR proteins. For example in the regulation of src pre-mRNA splicing, SF2/ASF interacts with a negative regulator of splicing (NRS) to form a repressive complex upstream of the src exon and causes the skipping of the exon (McNally and McNally, 1996). In this complex, it is not clear which protein plays a more important role on the splicing repression. Interacting partners could be another layer of the splicing regulation by SR proteins, just as was found in the case of pre-mRNAs regulated by Fox and Nova.

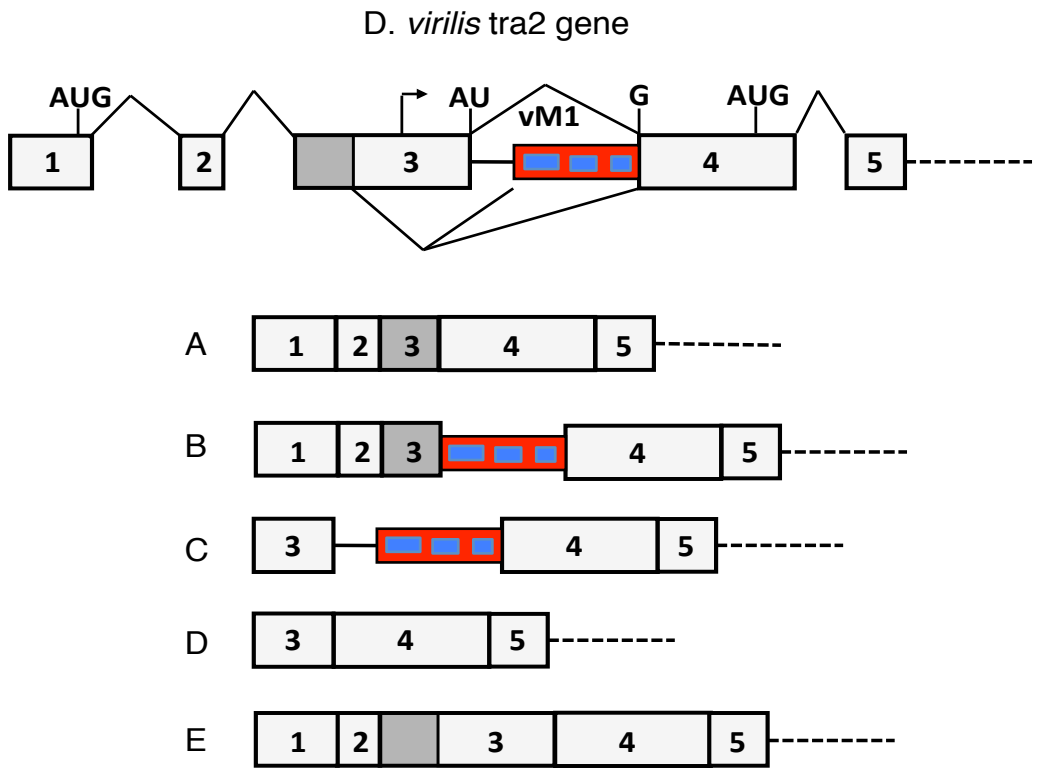


Figure 1-15. M1 intron retention in *Drosophila virilis*

The M1 intron in *Drosophila virilis* is also regulated by Transformer 2. The Tra2 gene from D. *virilis* is shown with gray boxes and horizontal lines corresponding to the exons and introns respectively. Red boxes represent the retained part of the vM1 intron. Blue bars represent repeated binding sites for the Tra2 protein. Tra2's binding to the repeats will repress vM1 intron splicing resulting in Type C transcripts but it also facilitate utilization of a 3' splice site available upstream of the binding site within the vM1 intron, causing use of a small intron upstream of the binding region and production of Type B mRNA.

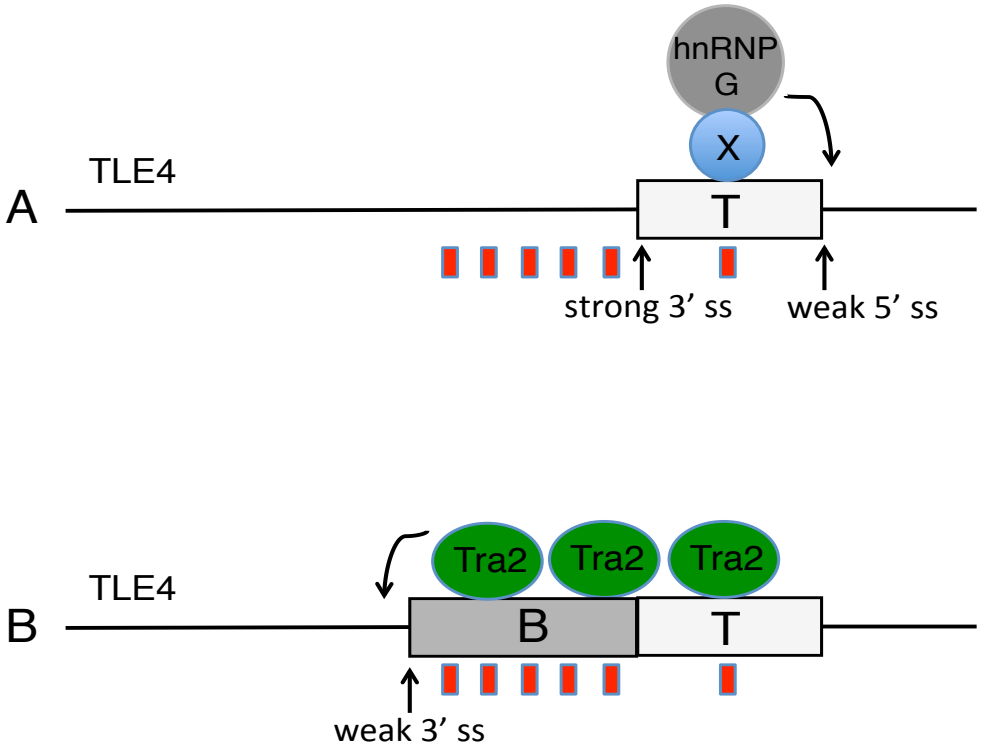


Figure 1-16. TLE4 alternative splicing regulated by hnRNP G and Tra2

Without Tra2 overexpression (A), testis-specific exon T is included by hnRNP G complex with other factors recognizing the weak 5’ splice site. With Tra2 protein level increased (B), a longer exon is included in the testis by Tra2’s binding to the repeats upstream of the exon T and recognizing the weak 3’ splice site. Red boxes indicate the Tra2 binding sites.

5. RNA splicing and germ line development

As a critical cellular process, alternative splicing is involved in many developmental events (Kalsotra and Cooper, 2011). Its disruption can cause developmental defects or diseases including cancers (David and Manley, 2010). However how alternative splicing regulates germ cell development has not been extensively studied and well understood.

As a highly organized system, lots of genes are alternatively spliced during gametogenesis including those involved in sex determination, signal transduction and meiosis. Under normal conditions, some genes have multiple spliced isoforms (Liu et al., 2011) and some of these isoforms are testis- or ovary- specific (Chuman et al., 2009; Daniel et al., 2001). There is some evidence showing that RNA splicing can be used by germ cells as a way of adapting to external conditions (Daniel et al., 2001). For example, a mouse gene AchE (acetylcholinesterase) has many variants differing in its C terminus produced by the alternative splicing. A splicing variant AchE-R was reported to interact with RACK-1 (receptor of activated protein kinase C) and probably induced apoptosis in germ cells under stressful conditions. AchE-R is also able to interact with enolase and elevate sperm motility by increasing its metabolism (Mor et al., 2008).

Defects in splicing are also associated with human infertility. It is known that splicing defect due to the mutations in intron 9 of Wilm's tumor gene 1 (WT1) can cause decreased expression level of SRY and SOX9, which is responsible for Frasier syndrome (Schumacher et al., 2008). Also a point mutation in the

splice acceptor site of intron 10 of LH receptor gene is reported to be responsible for male hypogonadism (Bruysters et al., 2008). In Drosophila, a defect in the splicing of Dic61B, a dynein intermediate chain gene, can cause abnormal assembly of the sperm axonemal complex (Fatima, 2011). Although these studies identified specific splicing defects, they put more emphasis on the aspect of developmental consequences caused by the impairments of RNA splicing. But the regulatory mechanisms of those alternative splicing events have not been elucidated.

Very little is known about how germline alternative splicing events are regulated. In only a few studies have critical RNA elements and splicing regulators been identified or their relationship studied. Human Tra2 has been shown to play roles in some of these splicing events. For example, human HIPK3 (homeodomain interacting protein kinase 3) is localized in PML bodies which are related with p53 phosphorylation and apoptosis. Its testis-specific splicing was regulated by several well-known splicing regulators, such as hnRNP A1, Tra2, ASF/SF2 (Venables et al., 2005). ASF/SF2 can form a complex on the testis-specific exon while Tra2 and hnRNP A1 will compete with each other to access the complex. When Tra2 was hypophosphorylated, it was found to form a highly stable complex with SF2 and SRp40, and promote the recognition of the weak 5′ splice site of the testis-specific exon. In another substrate TLE4 (Transducin Like Enhancer of split 4) (Liu et al., 2009), hnRNP G-T and RBMY can bind to a testis-specific exon which is within intron 6 and normally spliced out with the intron in other tissues, then activate its splicing in

human testis (Figure 1-16). These studies revealed details of the mechanisms of alternative splicing events that happen in testis. However their focus is limited to the regulation of alternative splicing at the molecular level, with little exploration of how important these molecular events are for the normal development of germ cells.

5.1 The role of Tra2 in spermatogenesis

As a splicing regulator that is required for sexual differentiation in somatic cells, Tra2's function in testis is also important. Homozygous mutant tra2 flies are sterile and their seminal vesicles are empty. Belote showed that Tra2 is required for normal spermatogenesis (Belote and Baker, 1983). In tra2 mutants, the spermatid heads were not able to elongate, but formed in a dense round shape compared with the needle-shaped heads in wild type (Belote and Baker, 1983). Some preliminary data suggests that mutant primary spermatocytes underwent a delay in entry to meiosis but eventually complete normal meiotic divisions (unpublished data, Unni and Mattox). It is not known if the delay results from changes in the RNA splicing or if it is responsible for infertility.

Studies on the auto-regulation of Tra2 expression in the germline suggest that splicing of the M1 intron is required for normal germ cell development (Mattox et al., 1990). As I discussed in section 4, this negative autoregulation is believed to limit the amount of the functional Tra2 isoform, Tra2-PC. That too much Tra2 is also toxic for germ cell development was demonstrated by deploying a modified system that could escape negative

autoregulation and elevate Tra2 level in the meantime (Mattox et al., 1996).

As a well-established model, the study of Tra2 autoregulation could be very useful to understand the mechanism of alternative splicing in the germline. More importantly, it will be powerful to test the effects of splicing events on germ cell development. Genetic and cytological analysis can be used to characterize the phenotypes and identify molecular targets downstream of Tra2 in which RNA splicing may be affected.

6. Goals of this study

Although SR splicing factors have been studied extensively for many years, their ability to repress splicing has remained obscure and the mechanisms unclear. As I discussed in section 3, such dual roles are not limited to SR proteins but are also observed in some hnRNP splicing family members. And it is now clear that the positions of regulators binding in relation to splice sites play a critical role.

The Tra2 protein provides an intriguing example of these dual roles for its positive role in dsx splicing and the negative role in M1 intron retention. What is more interesting is that these opposite activities have also been shown to be position dependent. The ISS element within M1 intron can mediate Tra2-dependent activation or repression depending on whether it is positioned in an exon or an intron (Qi et al., 2007; Shen and Mattox, 2012). Moreover molecular tethering experiments demonstrate that activation and repression functions are mediated by distinct and

separable effector domains of the protein (Shen and Mattox, 2012). The RS domains of Tra2 are required for the positive splicing activity that occurs from exonic positions, while the RRM domain is critical for the negative activity observed from intronic sites. The RRM domain here is not thought to be used to bind RNA elements because Tra2 was tethered on RNA by the MS2 coat protein in these experiments. It is possible that the RRM could interact with some other proteins to help Tra2 repress M1 intron splicing. Since different domains are responsible for its distinct regulatory activities, it will be interesting to know what factors are the partners of Tra2 and what kind of complexes are formed on the M1 intron to make intron splicing repressed. To explore this novel activity I will use an RNAi screen to identify potential Tra2 co-repressors and characterize their activities during M1 intron splicing. My work reveals that Half pint, a conserved RNA binding factor previously described for its role in the positive regulation of splicing, plays an essential role in the splicing repression by Tra2.

Negative regulation of splicing by Tra2 is also important developmentally in the process of spermatogenesis. However the critical targets of Tra2 regulation in the germline have remained unclear. Most studies on the role of RNA splicing during spermatogenesis focused either on the cytological phenotypes and developmental consequences, without elaboration of the mechanism; or focused solely on the description of alternative splicing events without a clear developmental context. It is important to know how RNA splicing in normal conditions of germ cell development. In

other words, some detailed questions should be answered like: Specifically what critical target genes are alternatively spliced? How are these splicing events are regulated? What stages of germ cell development are affected by these splicing events? This type of approach will provide insights into the role of RNA splicing and spermatogenesis, and understand how the regulation of RNA splicing itself regulates germ cell development.

In the studies described here I will make use of the genetic approaches available in Drosophila to investigate how Tra2 and Half pint affect the alternative splicing of Taf1, a target that plays a vital role in spermiogenesis. This analysis provides insights into the role that splicing factors play in the development of the germline.

Chapter Two

An RNAi Screen for Factors Required in Tra2-Dependent Repression of pre-mRNA Splicing

Introduction

The mechanisms responsible for repression of splicing by SR factors are poorly understood. To shed light on these mechanisms it is necessary to identify factors that collaborate with SR factors specifically in the repression but not the activation of splicing. Transformer 2 provides a classic example of splicing repression by an SR splicing regulator. It has been found responsible for the retention of M1 intron in its own pre-mRNA in Drosophila male germline. Previous studies have focused primarily on cis acting RNA sequences involved in repression. Several such elements within the M1 intron and the upstream exon are necessary for Tra2-mediated splicing repression (Figure 2-1). For example an exonic splicing enhancer within exon 3 was shown necessary to obtain maximal M1 repression but was not sufficient to confer repressions on other introns (Chandler et al., 2001). An intronic element close to the 5′ splice site was shown to function as an intronic splicing enhancer (ISE), which promotes efficient basal M1 splicing in the soma where repression is not observed in most cell types (Chandler et al., 2001). More recently an intronic splicing silencer (ISS)

sufficient to confer Tra2 dependent repression on another intron was identified (Qi et al., 2007)(Figure 2-2A). This 78 nucleotide element is located immediately upstream of the predicted branch point of the intron. Within this ISS, there are five repeats of the sequence CAAGR that are required for Tra2 binding. In in-vitro studies, when the intact ISS was inserted into ftz intron, it was found to be sufficient to repress the intron splicing by Tra2 protein. When these repeats were progressively mutated, both Tra2 binding and the efficiency of repression were diminished in a manner related to the number of repeats affected (Qi et al., 2007).

The binding of Tra2 was found to inhibit the formation of spliceosome complex A. The mechanism of M1 retention by Tra2 could be simply explained if Tra2's binding to the ISS located upstream of the branch point physically blocks the access of U2 snRNP to the pre-mRNA, an essential step in the formation of this complex (Figure 2-2B). However when the ISS was moved upstream away from the branch point, about 30 nucleotides distant, it still can mediate the splicing repression by Tra2 protein (Qi et al., 2007). That suggests instead of passive occupation of the branch point and occlusion of U2 snRNP, Tra2 binding with the ISS actually forms a functional repressive complex that actively interferes with spliceosome assembly.

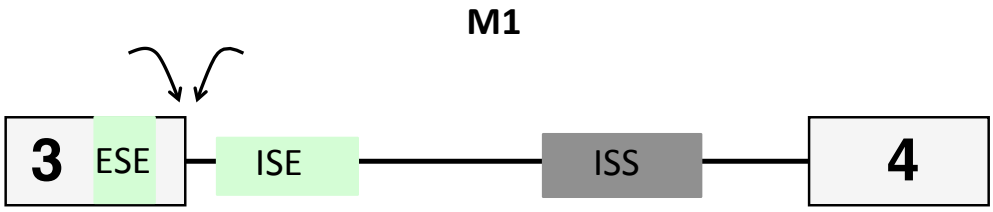


Figure 2-1. Diagram of the regulatory elements within and near M1 intron

The section of tra2 pre-mRNA structure is shown with boxes and lines, which correspond to exons and intron respectively. Darker gray box represents the intronic splicing silencer. The green boxes represent the enhancers within exon 3 and M1 intron. An intronic splicing enhancer within M1 intron and an exonic enhancer in exon3 contribute to the recognition of the 5′ splice site.

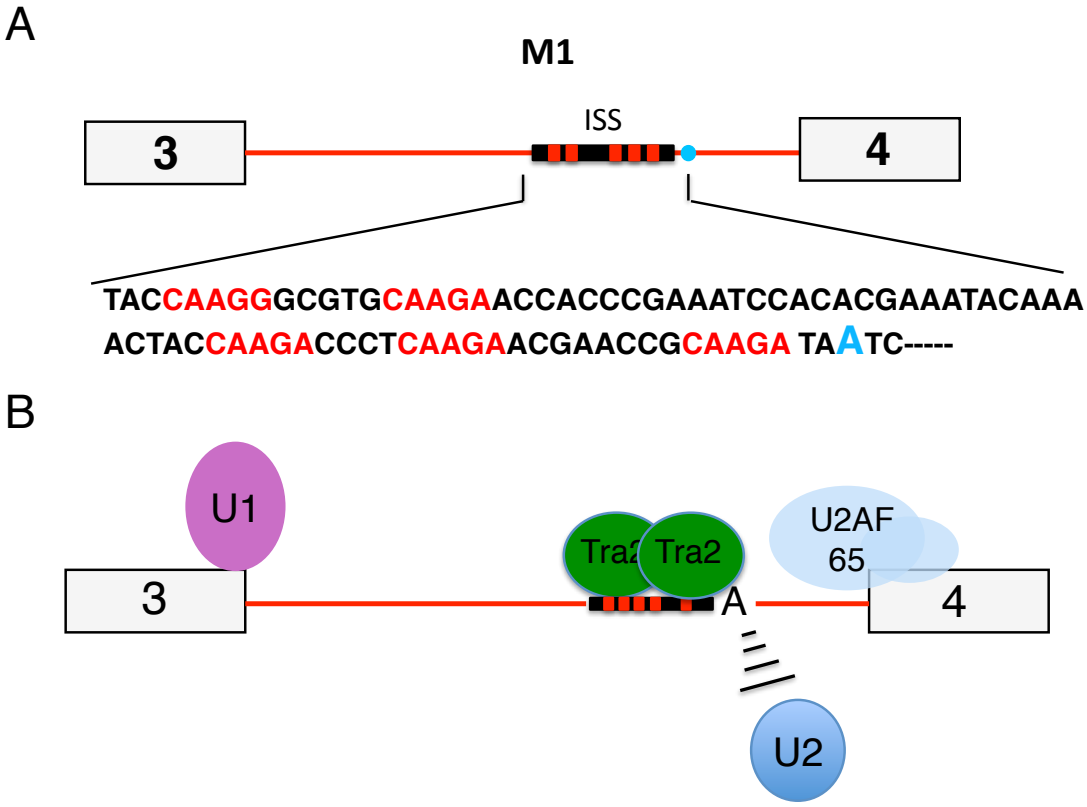


Figure 2-2. A model for M1 retention mediated by Tra2

(A) The diagram of partial tra2 RNA and ISS element (indicated by the thick black bar and five red small bars). The ISS sequence is shown below the diagram with the binding repeats labeled with red color. The predicted branch point is shown with blue "A". (B) Tra2 was thought to occupy the ISS element and prevent U2 snRNP from binding to the branch point site, thus repress functional spliceosome complex formation.

This repressive complex is also suggested to be different from another Tra2-dependent complex that activates dsx splicing in the soma of Drosophila females. First, Tra2 binding sites are different in these two complexes. Tra2 binds directly to a TCAACA element within dsx exon 4 (Lynch and Maniatis, 1996), while it binds to CAAGR in M1 intron. Second, Transformer (Tra), another key component in the splicing complex of dsx exon 4, is not needed in M1 retention, because Tra is expressed only in females. When Tra was forced expressed in male germline, no effect on M1 splicing was observed (Chandler and Mattox, unpublished). Third if ISS was replaced by just CAAGR repeats, most of its repressive function was lost (Qi et al., 2007), while dsx repeats appeared to function well without other elements (Hertel and Maniatis, 1998). Fourth, the active and repressive functions of Tra2 are separable. The RS domain of the protein has been shown to be the biochemical effector region responsible for the activation of splicing from exonic positions, but its RRM acts as the effector of its repressive activity when bound with an intron, as discussed in Chapter One (Shen and Mattox, 2012). These observations suggest that distinct factors collaborate with Tra2 during activation and repression of splicing and that the repression is likely to depend on one or more proteins that have not been identified as in classical studies on dsx activation.

To identify Tra2 co-repressors, we carried out a small-scale RNAi screen in S2 cells with a luciferase signal-based M1 splicing reporter. This screen identified several factors that are strong candidates to function with Tra2 in splicing repression.

Materials and Methods

Plasmids and primers

pM-Luc (M1 reporter) was constructed based on pBluescript SK+ vector (pSK+). The promoter from actin 5C was amplified and ligated into pSK+ after KpnI and ApaI digestion. The polyadenylation element from SV40 was amplified and ligated into pSK+ after SacII and SacI digestion. A segment of the tra2 pre-mRNA including the entire M1 intron and flanking exon sequences was amplified and ligated into pSK+ after ApaI and BamHI digestion. Luciferase coding sequence was amplified and ligated into pSK+ after BamHI and XbaI digestion. The primers used are listed below:

actin 5c: 5-ATGCCCTACTAGAAGATGTGT, 3-CTCAAACGGTAGTGATATGAA;
tra2 RNA: 5-TTTCATTTGGATTTGCCCCCT, 3-TTCGCGATCGCGTGATGAACG;
luciferase cDNA: 5-GAAGACGCCAAAAACATAAAG, 3-TTACACGGCGATCTTTCCGCC;
SV40 signal: 5-GATCATAATCAGCCATACCAC, 3-GATCCAGACATGATAAGATAC.

3XFlag Tra2-PC was made by inserting a 3XFlag tag into the N terminus of the 6XHis Tra2 PC baculovirus expression construct (Qi et al., 2007). The DNA fragment encoding the 3XFlag tag was synthesized by Integrated DNA Technologies.

Tra2-PC cDNA was amplified from pFastBac 3XFlag Tra2-PC and then inserted into pSK+ between the Actin5C promoter and an SV40 polyadenylation signal (pSK-AS).

pftz-Luc was constructed with the similar way of pM-Luc. The only difference between these two reporters is that the ftz DNA containing the ftz intron and flanking exon sequences, was amplified with ftz primers:
ftz RNA: 5-ATGGACTACTTGGACGTCTACTCG, 3-CTTGATCTGCCTTTCGCTCAG.

RNA interference library

RNAi library was generously provided by Dr. Eric Wagner. Briefly, the library contains cDNAs of CDS fragments of 247 RNA binding proteins in Drosophila. The RNAi library is amplified first, then 10μL PCR products was used as template to synthesize single strand RNA by in vitro transcription (Ambion T7 MEGAscript kit). In vitro transcription was performed at 37°C for over night. 1μL DNAseI (NEB company) was added to each well, incubated at 37°C for 20 minutes. Then 100μL dsRNAs were made by mixing equal volume of both sense and antisense single strand RNAs in the dsRNA buffer (100mM NaCl, 20mM Tris-HCl, pH 8.0, 1mM EDTA) heated at 100°C, cooled down at room temperature for at least 30 minutes, and finally incubated on ice before use. Both DNA templates from PCR reactions and RNA products from in vitro transcription were validated by the gel electrophoresis. The library was stored at -80°C.

RNAi screen in S2 cells

$1X10^7$ S2 cells were seeded within 10cm dishes. 30ug total DNA, including a Renilla luciferase reporter plasmid as a control for transfection efficiency, were transfected with 60μL cellfectin (Invitrogen). After 6-7 hours, fresh medium was changed, cells were counted and reseeded into 96-well plates with $3X10^4$ cells per well. 1.5μL dsRNAs were added into the wells and the plates were incubated at 28°C for 72 hours.

Luciferase assay

The medium in each well of RNAi treated cells\ was removed and 20μL passive lysis buffer (Dual-Luciferase reporter Assay system from Promega) was added. This mixture was incubated at room temperature for 15 minutes. Luciferase Assay Reagent II (LAR II) was prepared by mixing lyophilized Luciferase Assay Substrate with Luciferase Assay Buffer II. 100μL LAR II was added into each well of 96-well plates and firefly luciferase activity was measured on a Perkin Elmer VICTOR™ X5 Multilabel Plate Reader. Then another 100μL Stop&Glo reagent was added, Firefly signal was quenched and Renilla luciferase activity was recorded.

Data analysis

Firefly luciferase activity was divided by Renilla luciferase activity for each sample. This ratio was used for further analysis. The results of luciferase activities from two independent experiments were collected and calculated by MA plot method.

Duplicate results for each well were calculated into two parameters: M and A. M = $\log_2$(N1/N2), which represents the reproducibility of the RNAi effect in that well. A = 1/2 X $\log_2$(N1 X N2), which represents the average value of luciferase activity in each well. N1 and N2 represent two values from each time of the screen. After each well has its own M and A values, they will be normalized by the M and A values from the negative controls, in which A value was set as 0(Liu et al., 2007).

Results

1. Validation of a splicing reporter system for the co-repressor screen

To identify factors that contribute to the Tra2-dependent repression of M1 splicing we designed a M1 reporter plasmid (pM-Luc) in which the expression of firefly luciferase depends on the splicing of M1 intron (Figure 2-3). The reporter plasmid includes part of tra2 gene that contains both the intact M1 intron and the flanking sequences from adjoining exon 3 and exon 4. A naturally occurring translation initiation codon is split by the M1 intron and is positioned as the only initiator that is in-frame with the downstream luciferase coding sequence. Cotransfection of Drosophila S2 cells with a plasmid expressing the Tra2-PC protein isoform results in increasing repression of splicing from this reporter and reduced luciferase levels (Figure 2-4). However, neither splicing nor luciferase activity from a control reporter (pftz-Luc) containing a constitutive intron from the ftz gene was repressed by the expression of Tra2-PC.

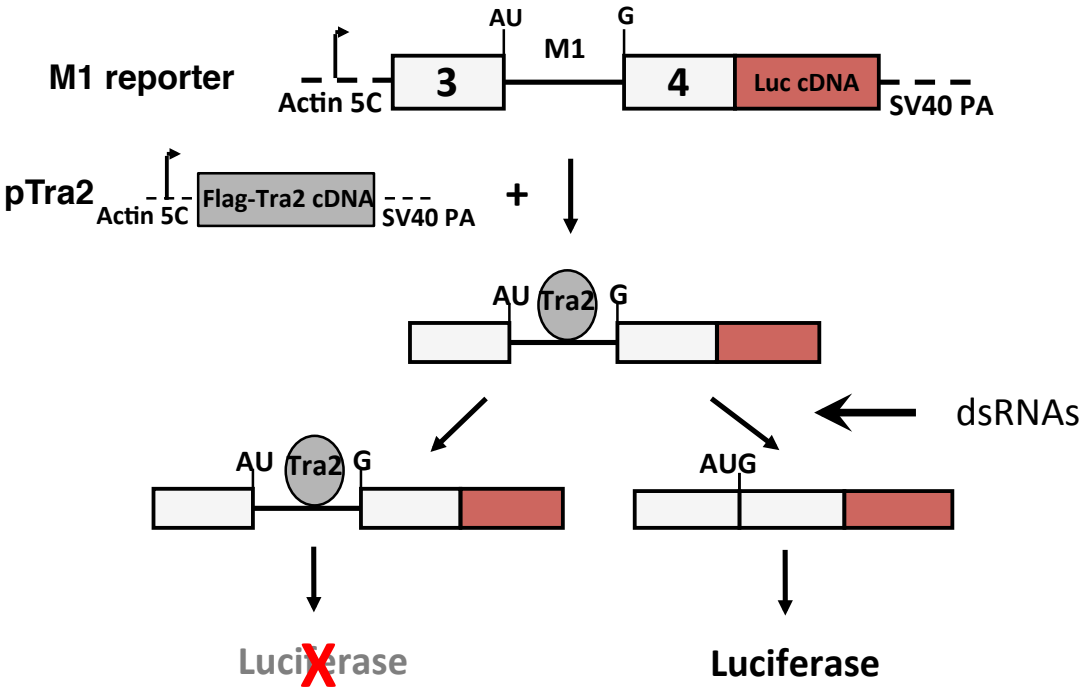


Figure 2-3. Scheme of RNAi screen in S2 cells

The organization of reporter plasmids used to screen for Tra2 cofactors in Drosophila S2 cells is shown. Splicing of the M1 intron from M1 reporter transcripts leads to expression of firefly luciferase (Luc). The exons and M1 intron derive from the endogenous Drosophila Tra2 gene. The initiation codon shown is naturally split by the intron and is in frame with the luciferase coding sequences. Construct of pTra2 has the same promoter and poly A signals with the M1 reporter. Cotransfection of both M1 reporter and pTra2 into S2 cells will cause minimum luciferase activity expression. While knockdown of any Tra2 corepressors in cells by its dsRNA will restore the luciferase signal.

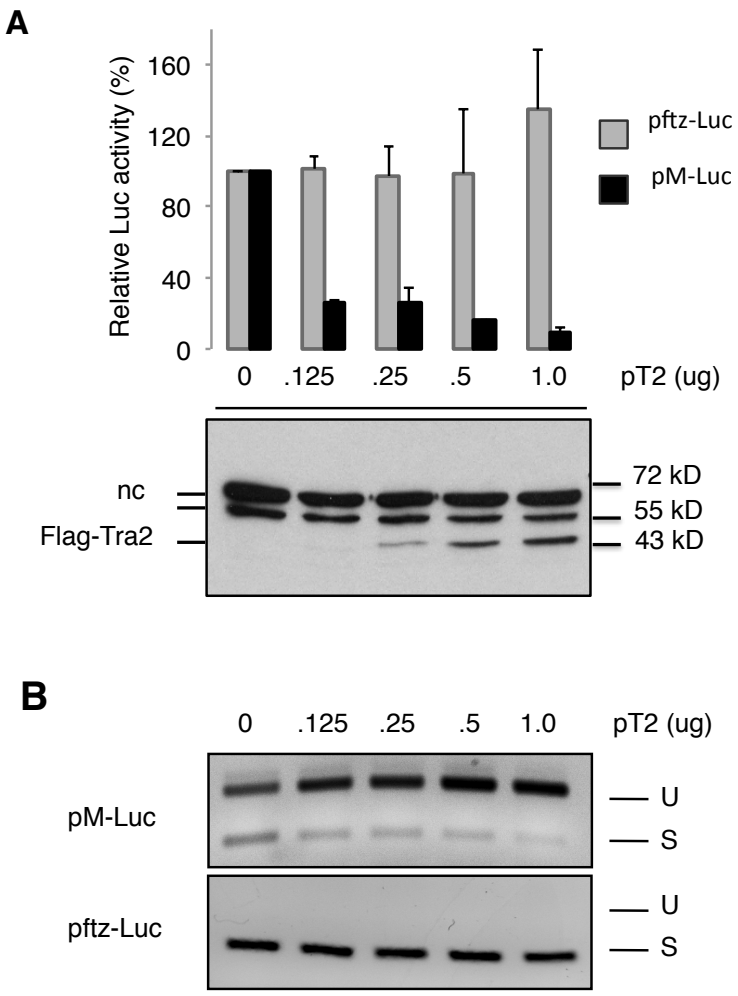

A
Relative Luc activity (%)
160
120
80
40
0
pftz-Luc
pM-Luc
0
.125
.25
.5
1.0
pT2 (ug)
nc
Flag-Tra2
72 kD
55 kD
43 kD
B
0
.125
.25
.5
1.0
pT2 (ug)
pM-Luc
U
S
pftz-Luc
U
S

Figure 2-4. M1 luciferase reporter detects specific repression of M1 splicing

(A) The luciferase activity from M1 reporter but not ftz reporter is repressed by the expression of Flag-tagged Tra2-PC in cotransfection experiments. Results from luciferase assays (graph) and immunoblots probed with anti-Flag antibodies are shown. The position of Flag-Tra2 and two nonspecific bands (nc) typically observed in such assays as well as molecular weight markers are indicated. (B) The effects Tra2-PC on the splicing of transcripts from both reporters, as detected by RT-PCR, is also shown. As expected, the ratio of amplification products from unspliced (U) to those of spliced (S) transcripts deriving from M1 reporter increases with higher levels of Tra2-PC.

This system was next tested for its response to dsRNA knockdown of Tra2 (Figure 2-5). Treatment with tra2 dsRNA restored the luciferase activity to nearly the same level as that of the reporter only. Treatment with U2AF50 dsRNA on the other hand dramatically lowered reporter activity. Since U2AF50 is a core basic splicing factor, its decreased level is expected to halt pre-mRNA splicing. This was indicated in Figure 2-5 by the lower luciferase activity that reflects less spliced transcripts. No effect was observed when treated with a non-specific control dsRNA (Figure 2-5). These results indicate that repression of the M1 reporter resulting from introduced Tra2-PC could normally respond to dsRNA treatments.

This system was further used to carry out a screen with a dsRNA library that targets a group of 247 drosophila proteins coding genes with known or potential roles in RNA metabolism (Park et al., 2004). The screening layout is shown in Figure 2-6. In each plate there are four kinds of controls included. Positive controls are the cells transfected with both M1 reporter and Tra2-PC, and treated with double stranded tra2 RNA, which is synthesized separately in our lab and distinct with the tra2 dsRNA found within the library.

From the screen we identified 15 candidate co-repressors that they had stronger effects on M1 reporter luciferase activities than the parallel controls treated with tra2 dsRNA.

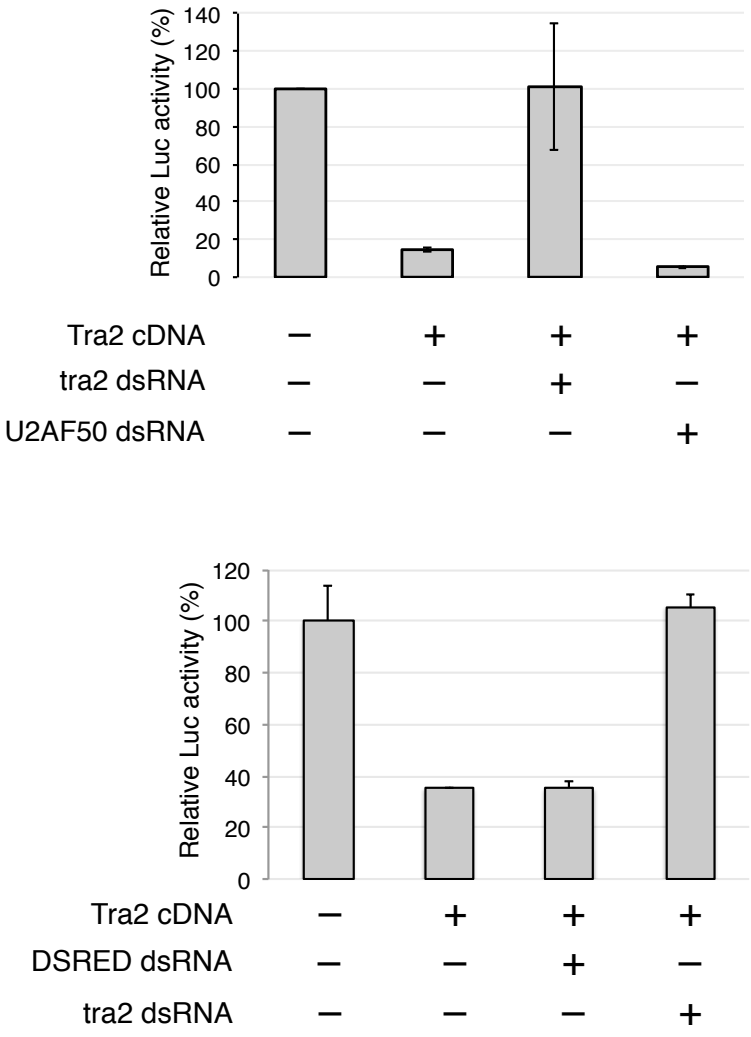


Figure 2-5. M1 luciferase reporter responds to dsRNA treatments

The luciferase activity from the M1 reporter is restored from Tra2-PC mediated repression by the treatment of tra2 dsRNA. No effect was observed when treated with the control dsRNA (DSRED dsRNA). However, even lower luciferase activity was observed when treated with U2AF50 dsRNA.

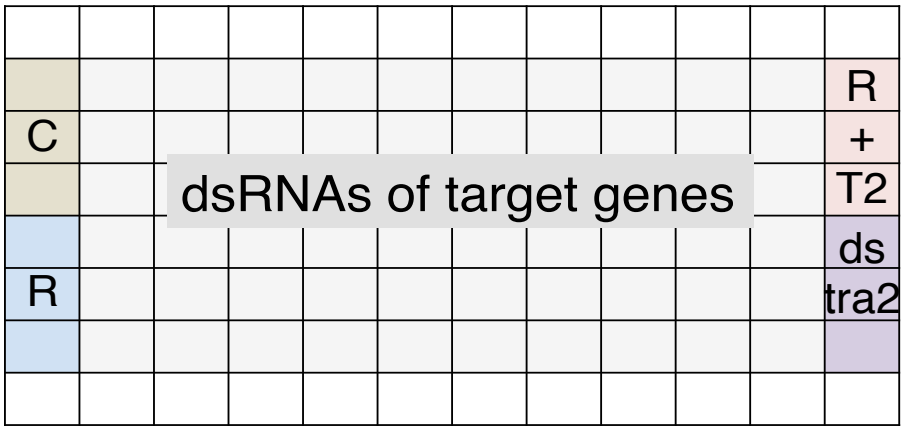


Figure 2-6. The layout of RNAi screen in 96 well plates

The layout of each 96 well plates for RNAi screen is shown. Wells in the gray middle part are the wells treated with the dsRNAs targeting 247 Drosophila protein-coding genes with RNA binding activity. In the wells of left side are the controls without transfection (C) and reporter only (R). In the wells of right side are the controls of without (R+T2) and with (dstra2) tra2 dsRNA treatment.

2. Results of RNAi screen

The RNAi screen was performed in duplicate. The F/R numbers of luciferase activities from each well were evaluated using an MA plot which relates the reproducibility of the results to the magnitude of change in the reporter activity. The final data is summarized in the plot shown as Figure 2-7.

In the plot, each dot represents one gene targeted by the dsRNA in the library. In X axis most dots fell around the value 0, which suggests most genes' dsRNAs have no significant effect on the M1 intron splicing. However all positive controls were found in the very right part of the X axis with the high A values as expected. In the Y axis, most dots were also found close to the value 0, which suggests most dsRNAs gave reproducible results.

By comparing with the mean value of positive controls, about 15 genes were identified that their luciferase activities were higher than the positive controls when their protein levels were knocked down by corresponding dsRNAs. Among the identified genes is tra2 which was blindly detected within the library as a positive candidate, supporting the reliability of the screen in detecting positives. The total targets were also arranged in their rank order of average luciferase activities, as shown in Figure 2-8, that the lowest is on the left and the highest on the right.

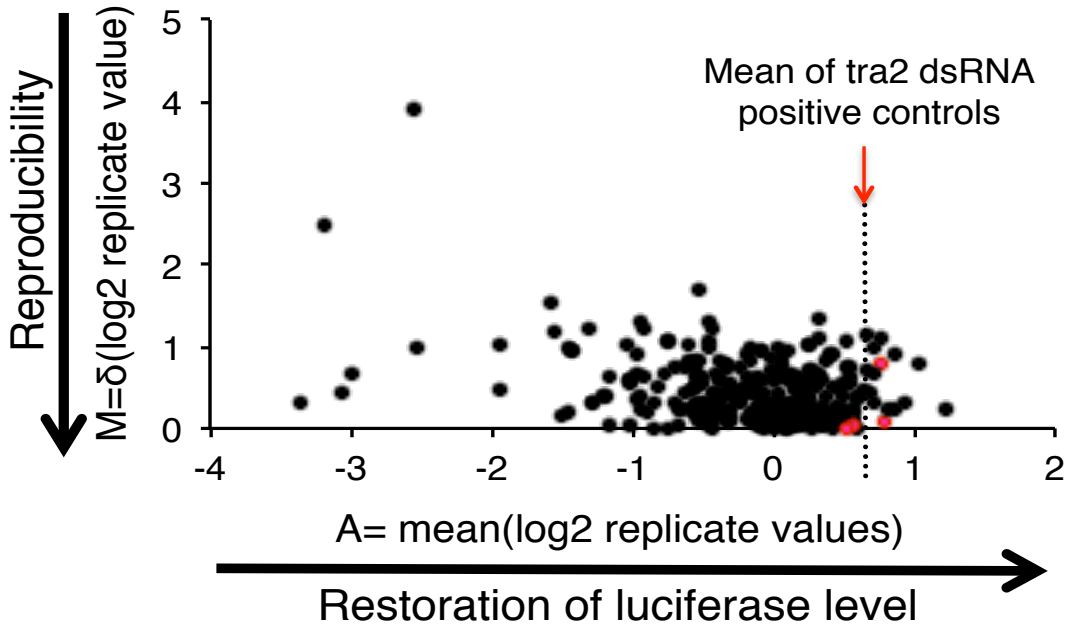


Figure 2-7. The result of RNAi screen

A library including dsRNAs that are targeting 247 RNA-binding protein coding genes of Drosophila was screened in duplicate by using the M1 reporter. Data was summarized in an MA plot. Candidates were identified with effects similar or greater to that observed with four of tra2 dsRNA controls (indicated as the red dots). Activity (A) indicates mean value of luciferase activities. Reproducibility (M) indicates the difference between log activities of the luciferase values from the duplicates. All values were normalized with internal control reporter expressing Renilla luciferase activity.

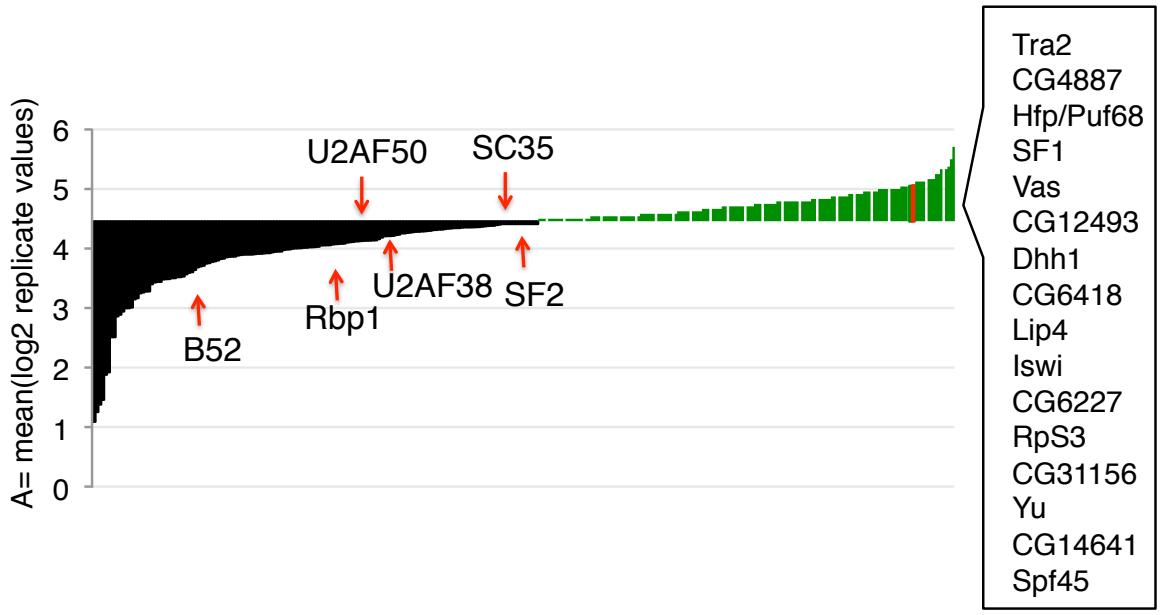


Figure 2-8. Distribution of library genes in the RNAi screen result

The finalized luciferase activities from all target genes in the library were displayed in the graph with the lowest luciferase number on the most left, and the highest on the most right. Positive candidates are listed on the right side of the graph. Other well-studied splicing factors are indicated by arrows within the graph. The red line represents the position of the positive control (dsTra2).

Among other 14 candidates, 8 of them, including CG4887, Hfp/Puf68, SF1, CG6418, Iswi, CG6227, CG14641 and Spf45, are all reported having activities in RNA splicing. CG12493 is a double-stranded RNA binding protein and is known to be required for Drosophila spermatogenesis. Vas, Dhh1, CG6418 and CG6227 all contain RNA helicase feature.

3. The validation of candidate co-repressors

To verify the results of the screen, the experiment was repeated with the identified candidates and negative controls for another two times using both the 96-well and 24-well plate formats. The final summarized luciferase results showed that only Hfp/Puf68, CG4887 and Spf45 had the most significant effects on the repression of M1 intron (Figure 2-9). No luciferase activity or lower luciferase activities were observed from the negative control targets of the screen.

Another way to validate the candidates is to test the effect of dsRNAs targeting them on the splicing of the M1 reporter. As shown in figure 2-10, a majority of reporter transcripts were spliced in the reporter only sample. This pattern was reversed and unspliced transcripts predominated when a plasmid expressing Tra2-PC cotransfected. Treatment with dsRNAs of CG4887, Hfp/Puf68 and Spf45 respectively decreased unspliced products significantly, as shown with RT-PCR and qRT-PCR (Figure 2-11). These results verified the results from the luciferase assay and suggest that these factors are likely to be co-repressors of Tra2 in M1 intron splicing.

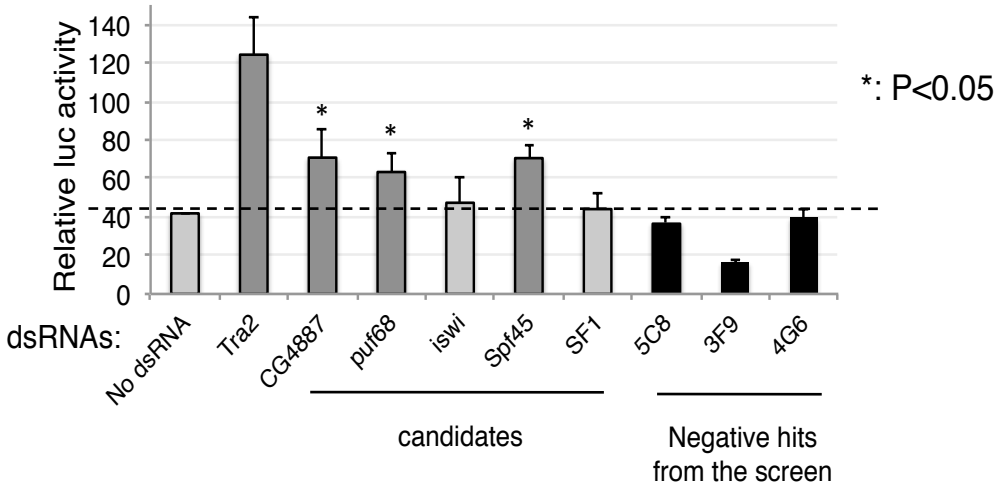
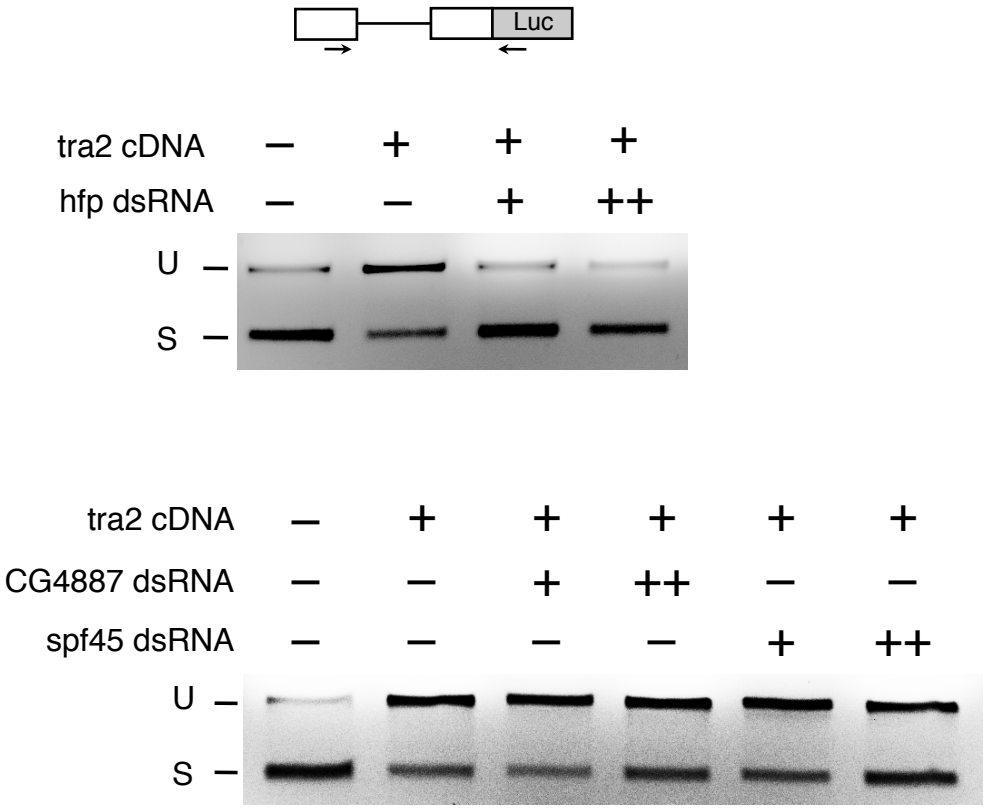


Figure 2-9. Validation of the positive candidates

The effects of dsRNAs targeting the positive candidates on the luciferase activity are summarized in the graph. Significant change in relation to the control of no dsRNA treatment is indicated (*). Control dsRNAs from the screen that showed no effect or decreased effect on the luciferase activity are shown in dark color.


Luc
tra2 cDNA – + + +
hfp dsRNA – – + ++
U –
S –
tra2 cDNA – + + + + +
CG4887 dsRNA – – + ++ – –
spf45 dsRNA – – – – + ++
U –
S –

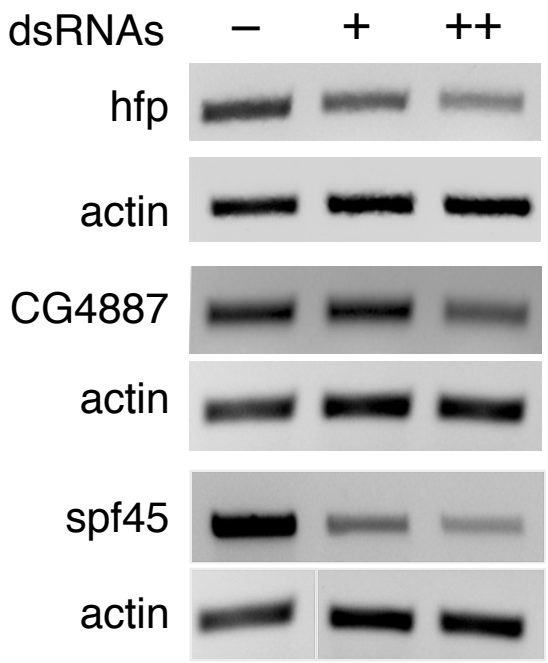

dsRNAs – + ++
hfp
actin
CG4887
actin
spf45
actin

Figure 2-10. The effects on splicing patterns of M1 reporter by dsRNAs of candidates

Splicing products from the M1 intron reporter were tested in various samples treated with the dsRNAs targeting positive candidates. The effects of the dsRNAs on M1 reporter splicing were shown in RT-PCR assay. Primer positions are indicated in the diagram. The mRNA levels of the candidates in S2 cells were also shown on the right side of the images.

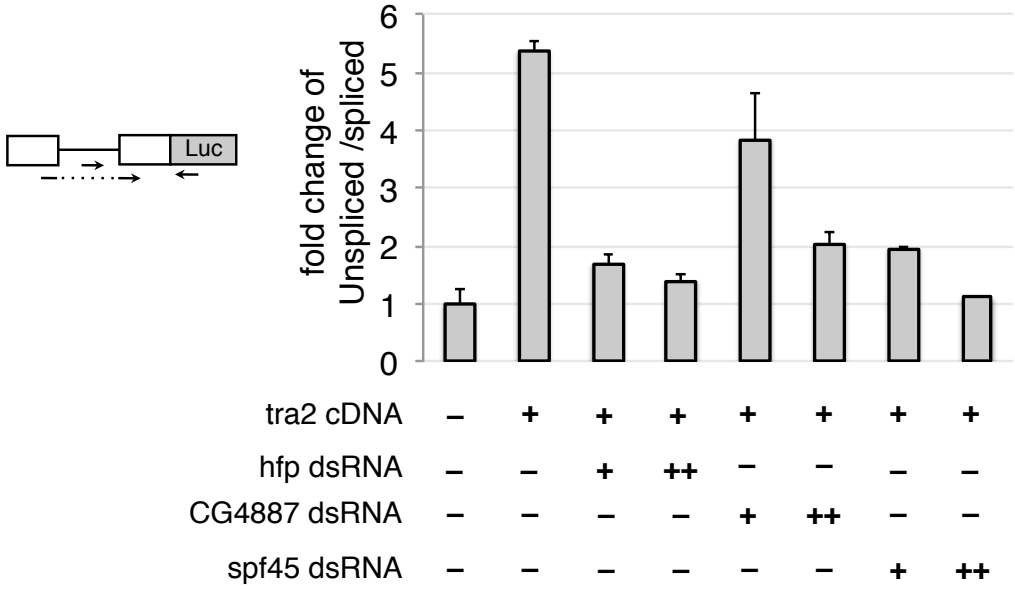


Figure 2-11. qRT-PCR assay on splicing patterns of M1 reporter by candidate dsRNAs

Splicing products from the M1 intron reporter were tested in various samples the same as that used in figure 2-10. The effects of the dsRNAs on M1 reporter splicing were shown with quantitative RT-PCR. Primer positions are indicated in the diagram.

Discussion

Using a small-scale RNA interference screen in S2 cells, I have identified at least 3 candidate factors that contribute to the Tra2-dependent repression of M1 intron splicing. To study the regulation of alternative splicing, people usually choose RT-PCR or Q-PCR method. However these methods are difficult to adapt to high-throughput screens due to the multiple manipulations required. On the contrary, a screen based on luciferase signal has been extensively used and accepted to identify factors involved in a particular molecular event. It can give real-time results, which avoids middle steps and also saves time. It is also quite sensitive, which is very helpful especially for the substrates with low splicing efficiency. A key feature of this study is the adaption of the luciferase assay to measure a particular alternative splicing event. Several previous studies of RNA splicing have also used splicing reporters based on luciferase activity. However few of them is based on a natural or specific splicing event (Gowrishankar and Rao, 2007; Younis et al., 2010).

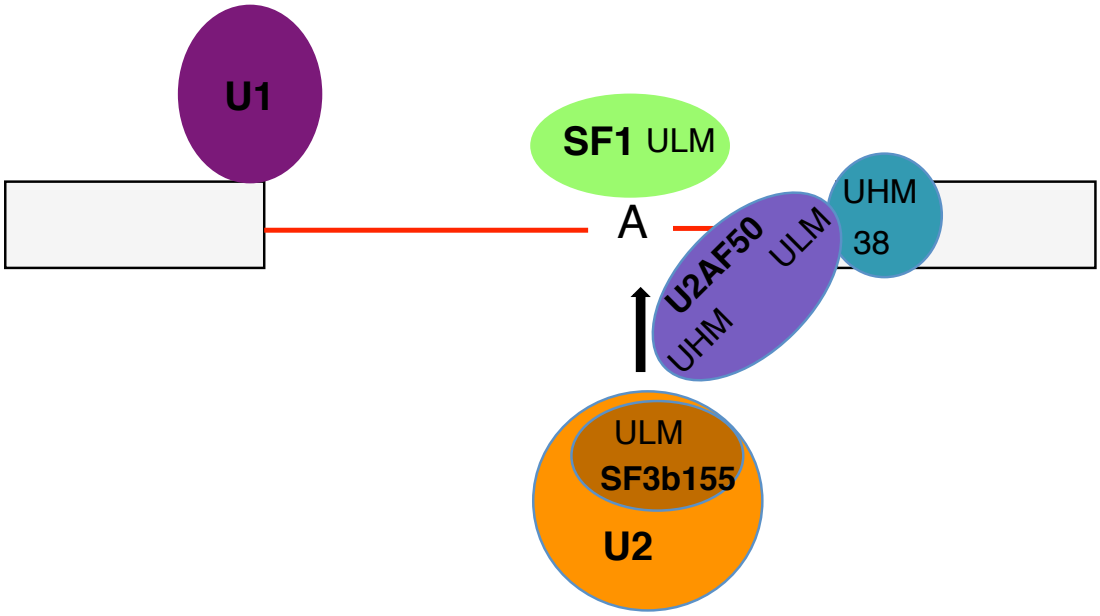


Figure 2-12. A model of interactions between UHM and ULM domains within the pre-spliceosome complex

Splicing factors with UHM and ULM domains recognize basic splicing signals within the 3′ region of an intron and communicate with each other by the UHM-ULM interactions, leading to pre-spliceosome formation and transformation into other splicing complexes. Briefly the interaction between U2AF50 and SF1 helps form the E complex. Then the interaction between U2AF50 and SF3b155 of U2 snRNP helps recruit U2 snRNP into the branch point, thus replace SF1 and transform the spliceosome to the A complex.

In this study, I make use of the special start codon split by the M1 intron in tra2 pre-mRNA. By fusing luciferase protein coding sequences downstream of exon 4 and in frame with this codon, I found it is possible to evaluate splicing performance based on luciferase activity. Thus this reporter is very compatible with a cell-level screen even in a genome wide scale. It can produce relatively reliable and quick outcomes telling us which are the potential candidates. It can narrow down the scale dramatically with less cost of money and time.

Several candidates identified in this study are known to play roles in alternative splicing events. Among them, Hfp/Puf68, Spf45 and SF1 share the similarity in that they each contain a U2AF homologue motif (UHM) thought important in several steps of RNA splicing. This structural domain is distinct, but related to the RNA recognition motif found in many RNA binding proteins. Functionally UHMs are thought to mediate specific protein-protein interactions, most particularly in proteins with ULM (UHM ligand motif) sequences. The UHM-ULM interactions play a prominent role in the formation of prespliceosomal complexes formed near the 3' splice site (Figure 2-12). For example SF1 binds to the sequence at the branch point and interacts with U2AF65 bound nearby at the polypyrimidine tract through the UHM-ULM contacts (Kielkopf et al., 2004). Further the UHM-ULM interactions occur between SF1 and SF3b155 as well as the large and small subunits of U2AF. The UHM of the splicing regulator Spf45 is also reported to be required in the alternative splicing of FAS pre-mRNA in vivo. The interaction between UHM of Spf45 and ULM of SF3b155, a component of U2 snRNP,

competes with the UHM-ULM interaction between U2 snRNP and SF1, leading to exon 6 skipping (Corsini et al., 2007). Hfp also contain a UHM domain in its C terminus. Since its human orthologue, PUF60 is known to recognize polypyrimidine tract sequences near the 3′ splice site of some introns, it is suggested to function in prespliceosome in a similar way as U2AF65 does (Hastings et al., 2007). A speculative model for the mechanism of M1 intron retention is that these candidate proteins, by utilizing their UHM domains, might interfere with the interactions between UHM and ULM domains in prespliceosome complexes and thus prevent A complex formation (Figure 2-13).

CG4887 is the Drosophila homologue of RBM5 (RNA binding motif protein) in mammalian organisms. Its function in Drosophila has not been studied very much, however as a tumor suppressor, RBM5 has also been reported to regulate alternative splicing of Fas and c-FLIP pre-mRNAs, which are the components in apoptosis pathway (Bonnal et al., 2008). Common substrates shared by splicing factors identified in the screen are summarized in Table 2-1. This suggests that the factors identified from the RNAi screen are probably functional related and that their shared effects on M1 intron splicing is not coincidental.

Genes	Homologous gene in mammal	Protein Domain	Known targets
Half-Pint/Puf68	PUF60	UHM	otu, Sxl
Spf45	SPF45	UHM	Fas, Sxl
CG4887	RBM5	OCRE	Fas, c-FLIP, Caspase-2

Table 2-1. The targets regulated and shared by positive candidates

The known targets for each candidate are shown, and common targets between the candidates are indicated with circles.

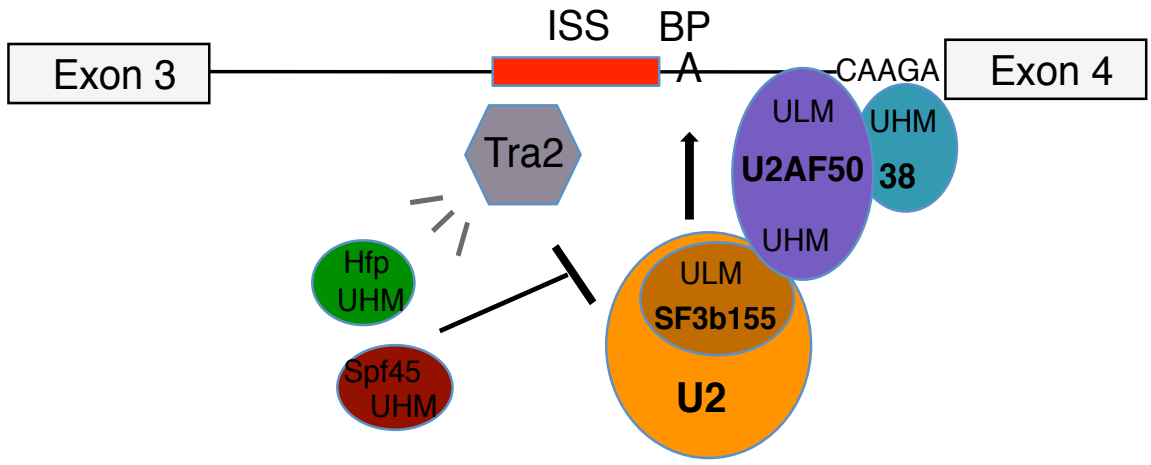


Figure 2-13. A model of splicing repression of M1 intron by positive candidates

Tra2 interacts with the positive candidates and form a complex on the ISS element. The cofactors with UHM domain will compete with U2 snRNP to interact with U2AF50 and prevent U2 snRNP from entering the complex E.

Other factors identified as possible corepressors correspond to helicases. CG6418 is annotated as the Drosophila homologue of DDX42 in mammals and Prp5 in yeast. As an RNA helicase it functions during the step of U2 snRNP recruitment into spliceosome. CG6227 is the homologue of DDX46 in mammals and Prp11 in yeast. It is thought to catalyze the conformational change of U2 snRNP and mediate the interaction of U1 and U2 snRNPs. Together with other RNA helicase identified in this screen, it will be interesting to investigate regulatory roles of RNA helicases during alternative splicing. RNA helicases are thought to catalyze rearrangement of RNA-RNA interactions and RNPs remodeling during RNA splicing, however as core splicing factors, whether they have regulatory roles is not well studied (Linder and Jankowsky, 2011).

It is interesting to see that a number of well-known splicing factors had no measured effect on the M1 intron splicing when they were knocked down, these include SF2 and SC35, as indicated in Figure 2-9. In previous study (Qi et al., 2007), Rbp1 was previously found to repress M1 intron splicing in vitro and observed to bind a sequence within the ISS, but its knockdown resulted in a decrease rather than an increase of the luciferase activity, which suggests a positive function instead of repression on the M1 splicing. Similar effects were observed in both U2AF50 and U2AF38, which is consistent with our understanding that U2AF factors are essential general splicing factors and usually promote RNA splicing. B52 is a Drosophila homologue of SRp55 in mammals. It is an essential splicing factor for Drosophila development (Kraus

and Lis, 1994). B52 can function together with SF2 in the regulation of alternative splicing of several substrates (Gabut et al., 2007). When B52 was knocked down in our system, luciferase activity became dramatically lower, which suggests B52 can actually promote M1 intron splicing in normal conditions perhaps due to a general role in splicing. Interestingly this result is consistent with previous observation from our lab. In an in vitro splicing assay, SR protein extract can antagonize Tra2's repressive function on M1 splicing. And the dominant component of SR protein extract is B52 (Qi, unpublished results). If and how B52 might antagonize Tra2's activity in M1 splicing is another interesting question to explore.

Chapter Three

Half pint is a Co-Repressor of Transformer 2

Introduction

Little is known about factors that collaborate with SR regulators in the repression of splicing. As described in the previous section we have used an RNAi based screening strategy to identify factors required for Tra2 dependent repression by the M1 intron. From a short list of candidates, we identified Half Pint/Puf68 (heretofore called Half pint, Hfp). Our focus on Hfp, was motivated by the fact that its mammalian homologue PUF60 is known to promote the utilization of weak 3′ splice sites and has been implicated in the control of alternative splicing. In addition, Hfp itself was reported to regulate the alternative splicing of several mRNAs in the Drosophila female germline (Van Buskirk and Schupbach, 2002). Notably, Hfp and PUF60 (also known as FIR) are multifunctional proteins that are known to act as transcriptional factors. PUF60 is also called FIR (FBP interacting repressor) and negatively regulates c-myc gene expression (Liu et al., 2000) through interactions with its far upstream sequence elements (FUSE). Independently PUF60 was identified as a splicing factor based on its function in recognition of the 3′ splice site (Page-McCaw et al., 1999). PUF60 also can function redundantly and sometime cooperatively with U2AF65 to regulate weak 3′ splice site recognition (Hastings et al., 2007).

Hfp also has similarity with proteins in UHM domain family of splicing factors (Kielkopf et al., 2004). The UHM domain has been found in several splicing factors including U2AF65, U2 snRNP subunit SF3b155, SF1 and Spf45. It is structurally like an RNA recognition motif but diverges in its RNP2 segment of the RRM. Instead of binding RNA, however, UHM domains are functionally suggested to be involved in protein-protein interactions. The interacting part of UHM domain is known as ULM domain. This domain features tyronine residues and is found in U2AF35 and U2 snRNP subunit SF3b155. The UHM-ULM domain interactions between several factors are thought to promote and stabilize pre-spliceosome complex assembly (Kielkopf et al., 2004).

Here I show that Hfp, which was regarded as a positive splicing regulator previously (Hastings et al., 2007; Page-McCaw et al., 1999), is able to help Tra2 repress M1 intron splicing in both Drosophila S2 cells and testis. Further I show the Hfp gene expresses two distinct protein isoforms that differ in their ability to repress M1 intron splicing.

Materials and Methods

Plasmids and primers

The M1 luciferase splicing reporter, Tra2-PC vector and ftz luciferase reporter are the same as used in Chapter Two.

pftz-ISS-Luc, pftz-mhc-Luc and pftz-ISS-mhc-Luc are all modified based on the pftz-Luc reporter. ftz-ISS sequence was amplified from pftz120 (Qi et al., 2007) and ligated into pftz-Luc by ApaI and XhoI digestion. pftz-mhc-Luc was made by three steps of PCR with mhc 3′ splice site sequence fusing into primers:

ftz RNA forward: 5-ATGGACTACTTGGACGTCTACTCG

mhc 3′ 1 reverse: 5- CTTGTTTGCAAGGGGATAAGTTCAATGGGTTAGCTAATGAGTTTT;

mhc 3′ 2 reverse: 5-

GTCTGACGGGTGCGTTTCGAGTCTTTGCAATCTTGTTTGCAAGGGGATAAG;

mhc 3′ 3 reverse: 5-

CTCGAGCTCCAGGGTCTGGTAGCGGGTGTACGTCTGACGGGTGCGTTTCGA.

pftz-ISS-mhc-Luc was made based on pftz-mhc-Luc with similar steps. Only the first step primer is different:

ISS-mhc 3′ 1 reverse: 5-

CTTGTTTGCAAGGGGATAAGTTCAAAAATAAGATTATCTTGCGGTTCG.

pFlag-Hfp68 was made in the same way as pTra2-PC, which has an actin 5c promoter and SV40 PA signal sequence. Hfp68 cDNA sequence was amplified from the plasmid purchased from the Drosophila Genome Resource Center. Flag sequences was inserted using a PCR based

strategy. pFlag-Hfp58 was made in the similar strategy with a different start position in the Hfp coding region.

Hfp68 cDNA forward: 5-ATGGACTACAAGGATGACGATGACAAGATGGGAAGCAACGACAGAGC;

Hfp58 cDNA forward: 5-ATGGACTACAAGGATGACGATGACAAGATGGAGCAGAGCATCAAGATG.

Hfp cDNA reverse: 5-CTAACCGGACAGATCTCCCTGATC.

HfpΔUHM reverse: 5-CTAGTCCACCGGCCGCATCAGTC.

Cell culture and transfection.

Dmel S2 cells was cultured in SFII-900 medium at 28 degree and split at regular intervals. When doing transfection, $2X10^5$ cells were seeded in 24-well plates. Totally 1.5ug DNA was mixed with 3μL cellfectin in 100μL medium. pSK-AS was used as the DNA carrier.

In vitro synthesis of double-stranded RNA

The procedure for dsRNA synthesis is like described previously (Park and Graveley, 2005). Briefly cDNA fragments was amplified and inserted into PCR4 vector (invitrogen). M13 reverse and M13 forward primers were used to amplify linear DNA template. Then Megascript T7, T3 and Sp6 kits (Ambion) were used to produce single-stranded RNAs. Single-strand RNAs were mixed in annealing buffer (100mM NaCl, 20mM Tris-HCl, pH 8.0, 1mM EDTA), put into 85 degree for 10 minutes and cool down at room temperature for at least 30 minutes, finally incubated on ice for use.

tra2 dsRNA primers: forward 5-CGGAATAGAAGTGGATGGTCG, reverse 5-TAGTTGCGGAGAGCGTGAAC.

Hfp3'UTR dsRNA primers: forward 5- TTAGAAGGGGGAGCTATCCG, reverse 5- GAATTGGAAACTATAGTTTA.

Hfp dsRNA primers: forward 5- GGTAGTGCCCACTCTTCCG, reverse 3-AAAATGATAGAACAAATGCGGG.

RNA interference assay

dsRNAs were added directly to the medium of SFII-900 with 4μg/well in 24-well plates, 20μg/well in 6-well pates. To get the maximum knockdown effect, dsRNA was added two more times in the next 24 and 48 hours points.

Luciferase assay

100μL Passive lysis buffer was added into each well of 24-well plates. 25μL was used to measure luciferase signal based on the manual from the Dual-Luciferase reporter assay system of Promega. And luciferase signals are measured on Perkin Elmer VICTOR™ X5 Multilabel Plate Reader.

RNA immunoprecipitation assay

$1X10^7$cells were seeded in $78cm^2$ dish. Total 30μg DNA were transfected. After 48 hours, cells were washed two times with 1XPBS buffer. Then cells were dounced repeatedly in RIP buffer (150mM NaCl, 50mM Tris pH 7.5, 5mM EDTA and 0.05% NP-40). Cell lysates were pre-cleaned with Sepharose Gammabind beads (Amersham) for one

hour. Cell lysates were then incubated with activated beads with or without conjugated anti-Hfp antibody at 4 degree for 4 hours. After washing beads for 3 times, the whole precipitates were treated with Trizol (Invitrogen). RNA isolated from precipitates was analyzed with RT-PCR.

Western blot

Cell was directly lysed in wells. The primary antibody concentration is 1:1000 for both anti-hfp antibody and anti-flag antibody (Sigma M2 monoclonal antibody). Secondary antibody and the ECL system were from GE company. After incubating one hour at room temperature, antibodies was washed 10 minutes at room temperature for three times. Then exposed to film.

RT-PCR

1μg RNA was used to do the reverse transcription following the manual of Superscript first strand RNA kit from invitrogen.

Real-time PCR

cDNA was synthesized with 1ug RNA total by Superscript first strand RNA kit. cDNA was mixed with 2X Syber Green PCR mix from ABI. Primer concentration is 0.625 ng/μL. PCR was performed in following conditions: 50°C 2 minutes and 95°C 10 minutes, followed by 95°C 15 seconds and 60°C 1 minute for 40 cycles.

S2 cell immunostaining and image analysis

S2 cells were seeded into 12-well plate with 5X10^5/well concentration. Coated covers (BD Biocoat) were put into the wells at the mean time. Cells were allowed to settle down over night. Plasmids were transfected next day. After 36-48 hours incubation at 28°C, cell medium was removed. Cells were washed once with 1XPBS, then fixed with 4% paraformaldehyde in PBS at 37°C for 30 minutes. After washing with PBS, cells were incubated in PBX (0.2% triton X-100 in PBS) at room temperature for 10-15 minutes. Blocking with 1% BSA at room temperature for one hour, then incubated with first antibody (1:200 in 1% BSA) at 4°C over night. Wash cells with PBS four times, then incubate in secondary antibody buffer (1:500 in 1%BSA) at room temperature for 1 hour. After four times washing step with PBS, take the coverslips out and put upside down onto the slides with mounting medium. Images were taken with Nikon Eclipse Ti confocal microscope. Images analyzed for nucleus/cytoplasm distribution by Imaris 7.3 after deconvolution by AutoQuant X3. The protein nuclear localization was calculated with the signal in nucleus divided by that in the whole cell.

Recombinant protein preparation

Full length Hfp68 cDNA was amplified from the vector AT08368 purchased from the Drosophila Genomics Resource Center (DGRC). Full length U2AF50 cDNA was amplified from whole fly RNA. Both of the cDNAs were inserted into pET49b+ vector and protein expression was induced by IPTG in BL21. Protein were purified by Glutathione

Sepharose (Amersham Pharmacia). 6XHis-Tra2 was expressed and purified from baculovirus (Qi et al., 2007).

The gel shift assay

Recombinant protein was incubated with 100pmol ^{32}p-labeled RNA in the binding buffer (25mM Tris pH 7.5, 25mM NaCl, 1mM EDTA, 0.1mg/mL tRNA, 0.5mg/mL BSA) at room temperature for 30 minutes. 2μL reaction product from total 10μL complex was loaded and separated on the polyacrylamide gel in 0.5 X TBE at 4°C for 3 hours. The gel was dried and set to exposure.

Results

1. Hfp is required for the Tra2-dependent repression of M1 intron splicing in S2 cells

The effect of Hfp dsRNA on the M1 repression was shown in Chapter 2 (figure 2-10). Knockdown of Hfp was found to dramatically restore the luciferase activity reduced by overexpression of Tra2-PC. The effect on luciferase activity is consistent with a de-repression of M1 splicing when Hfp is knocked down. Testing a second dsRNA targeting Hfp, we found similar effects on the luciferase activity and verified that levels were significantly reduced by the dsRNA, as shown by the immunobloting assay with anti-Hfp antibody. Luciferase expression was restored to over 60% the level observed in the absence of co-transfected Tra2-PC (Figure 3-1). The result from western blot showed that levels of Flag-Tra2PC expressed from the transfected cDNA were unaffected by Hfp knockdown indicating that the effect of Hfp knocking-down on M1 splicing is not due to the indirect effect from a decrease of Tra2 protein level (Figure 3-2). Taken together the above results indicate that Hfp contributes to the repression of M1 splicing in S2 cells.

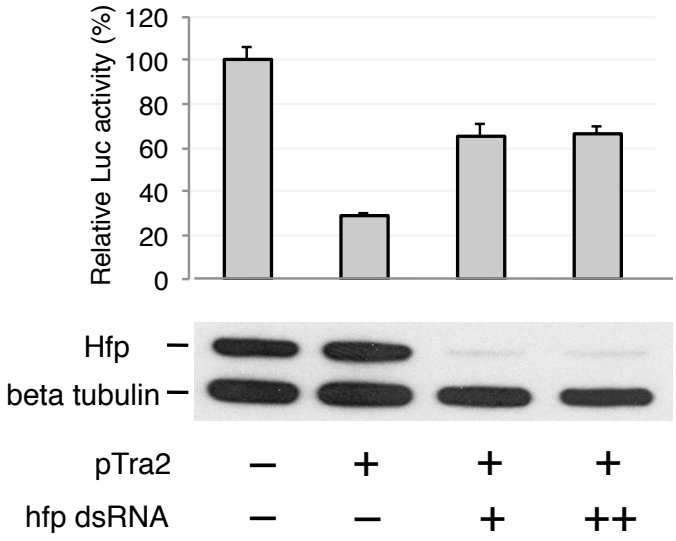


Figure 3-1. Luciferase activity is restored by Hfp dsRNA in S2 cells

The effect of Hfp dsRNAs on the luciferase activity from S2 cells transfected with both M1 reporter and Tra2 cDNA is shown. The graph shows percent luciferase activity in relation to that of the cells transfected with reporter only. Endogenous Hfp and tubulin protein levels from the same samples were analyzed with the western blot displayed below.

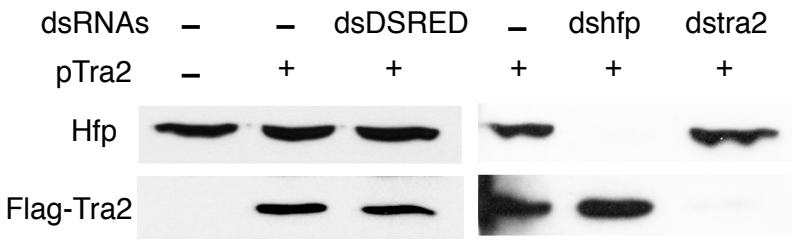


Figure 3-2. The Hfp dsRNA treatment has no effect on Tra2 protein level in S2 cells

S2 cells were transfected with pTra2 and were treated with Hfp dsRNA, Tra2 dsRNA and control dsRNA. Western blots detecting the protein levels of Flag Tra2 and endogenous Hfp are shown. A monoclonal anti-Hfp antibody was used to detect Hfp level. The M2 monoclonal anti-Flag antibody was used to detect the transfected Flag-Tra2.

2. Hfp associates with tra2 transcripts

The above effects on M1 reporter splicing could potentially result from indirect effects of other splicing factors that are regulated by Hfp. To test whether M1 RNA transcripts could associate with Hfp directly, RNA immunoprecipitation were carried out in S2 cell lysates with anti-Hfp antibody. RNA sequences from these precipitates were amplified by RT-PCR with primers inside the M1 intron and it was found that in comparison to the input signals a significant fraction of M1 transcripts are associated with Hfp, but no M1 signal was detected in beads only control (Figure 3-3A). Notably, the association occured independently of the increased expression of Tra2-PC indicating either that basal levels of Tra2-PC are sufficient to support Hfp binding or that Tra2-PC is not required.

To further confirm this association and test its specificity, M1 reporter was cotransfected with same amounts of ftz reporter and another GFP empty vector containing the same promoter and SV40 poly A signal (Figure 3-3B). The results showed tra2 M1 transcripts could be pulled down with the Hfp antibody. However no amplified products from the other two overexpressed transcripts were detected in the same precipitate. Together these results suggest that Hfp affects splicing of the M1 intron through specific associations with tra2 transcripts.

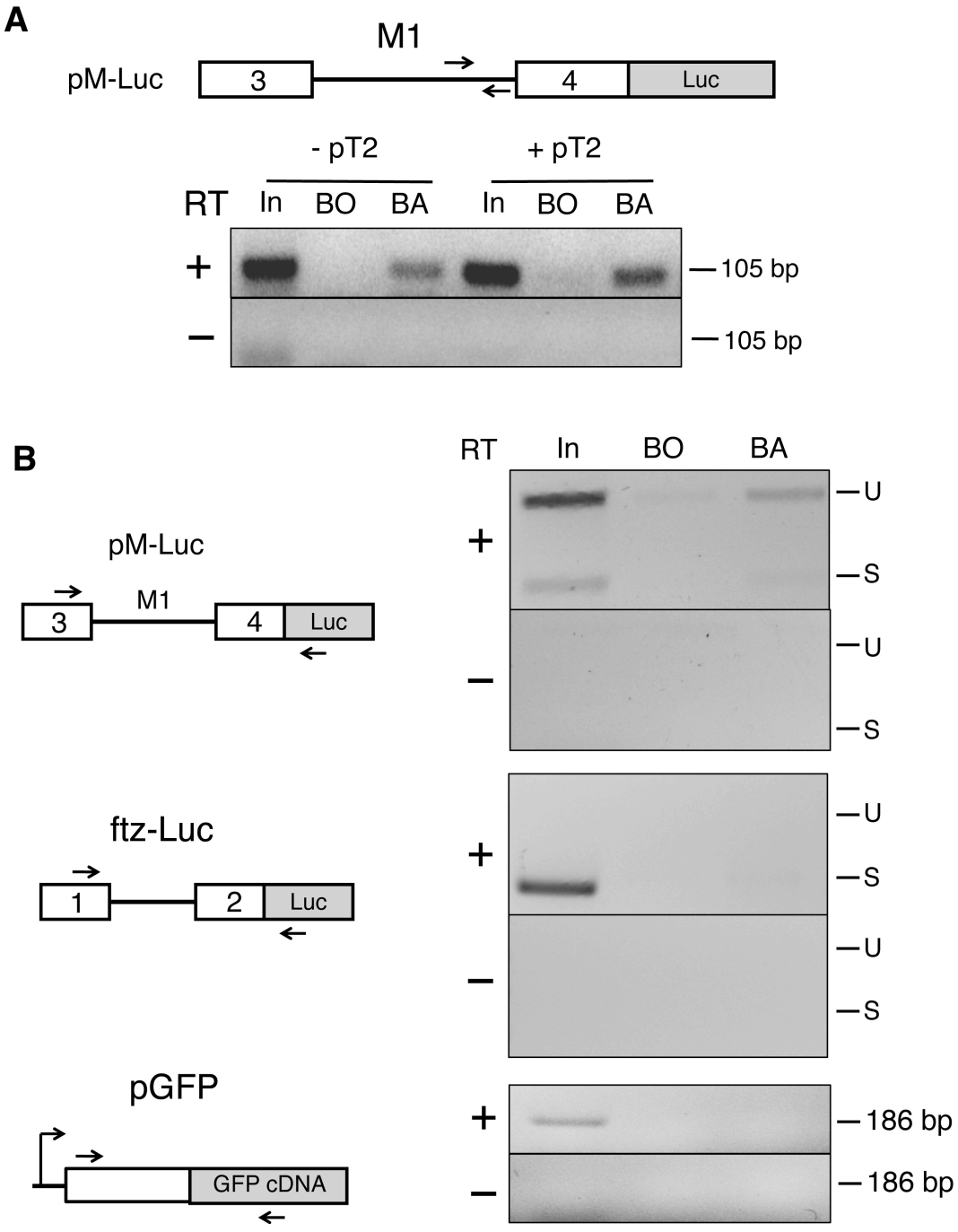

A
M1
pM-Luc
3
4
Luc
- pT2
+ pT2
RT
In
BO
BA
In
BO
BA
+
−
105 bp
105 bp
B
RT
In
BO
BA
pM-Luc
M1
3
4
Luc
+
−
U
S
U
S
ftz-Luc
1
2
Luc
+
−
U
S
U
S
pGFP
GFP cDNA
+
−
186 bp
186 bp

Figure 3-3. Hfp associates with tra2 transcripts in S2 cells

(A) S2 cells were transfected with the M1 reporter, at the same time with or without pTra2. RNA immunoprecipitation was carried out to pull down the transcripts with anti-Hfp antibody. RT-PCR assay detected RNAs precipitated with the Hfp antibody. Primers used are indicated in the diagrams. 20% input was used as controls. Expected RNAs were labeled with marker number on the right. In= input, BO=beads without antibody, BA=beads coupled with antibody. (B) S2 cells were transfected with three reporters of the same amounts. RNA immunoprecipitation experiment was done the same way as in part (A). Primers used for RT-PCR were indicated in the diagram. Association of Hfp with tra2 RNA is not dependent on Tra2 protein.

3. Both ISS and a weak 3′ splice site are required for the repression of M1 splicing mediated by Tra2 and Half pint

Hfp was previously known as a splicing activator that is involved in the regulation of 3′ splice site, however as a repressor, which elements in M1 RNA will be necessary for its negative function? Also it is interesting to know whether the sequences within M1 intron previously found to support the binding and repression by Tra2 are also sufficient for the repression by Hfp. Several elements in tra2 pre-mRNA have been reported to be involved in M1 repression (Chandler et al., 2001; Qi et al., 2007). Among them, an intronic splicing silencer (ISS) has been identified in vitro as a functional target of Tra2-PC as discussed in Chapter One and Introduction. In vivo, it has been shown that a weak 3′ splice site is critical for M1 retention in testis (Chandler et al., 2001). When the natural 3′ splice site of M1 intron was substituted with a Drosophila consensus sequence, M1 retention was abolished. Replacement with another weak 3′ splice site restored the retention to a wild type level.

As illustrated in figure 3-4, several recombinant luciferase reporters containing these different elements were made based on the backbone of ftz pre-mRNA which contains a single intron that is not normally regulated by Tra2. Notably ftz itself has a strong 3′ splice signal and no significant similarity to the ISS. The weak 3′ splice site from the intron E of myosin heavy chain gene was used to replace the 3′ splice site of ftz intron in the recombinant reporters.

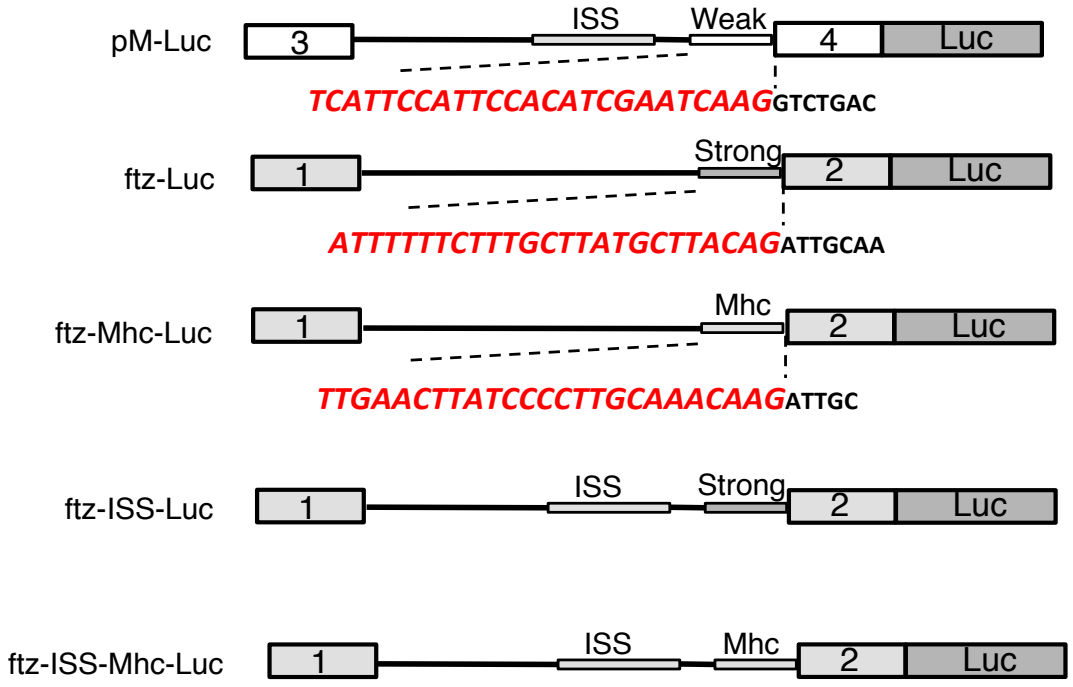
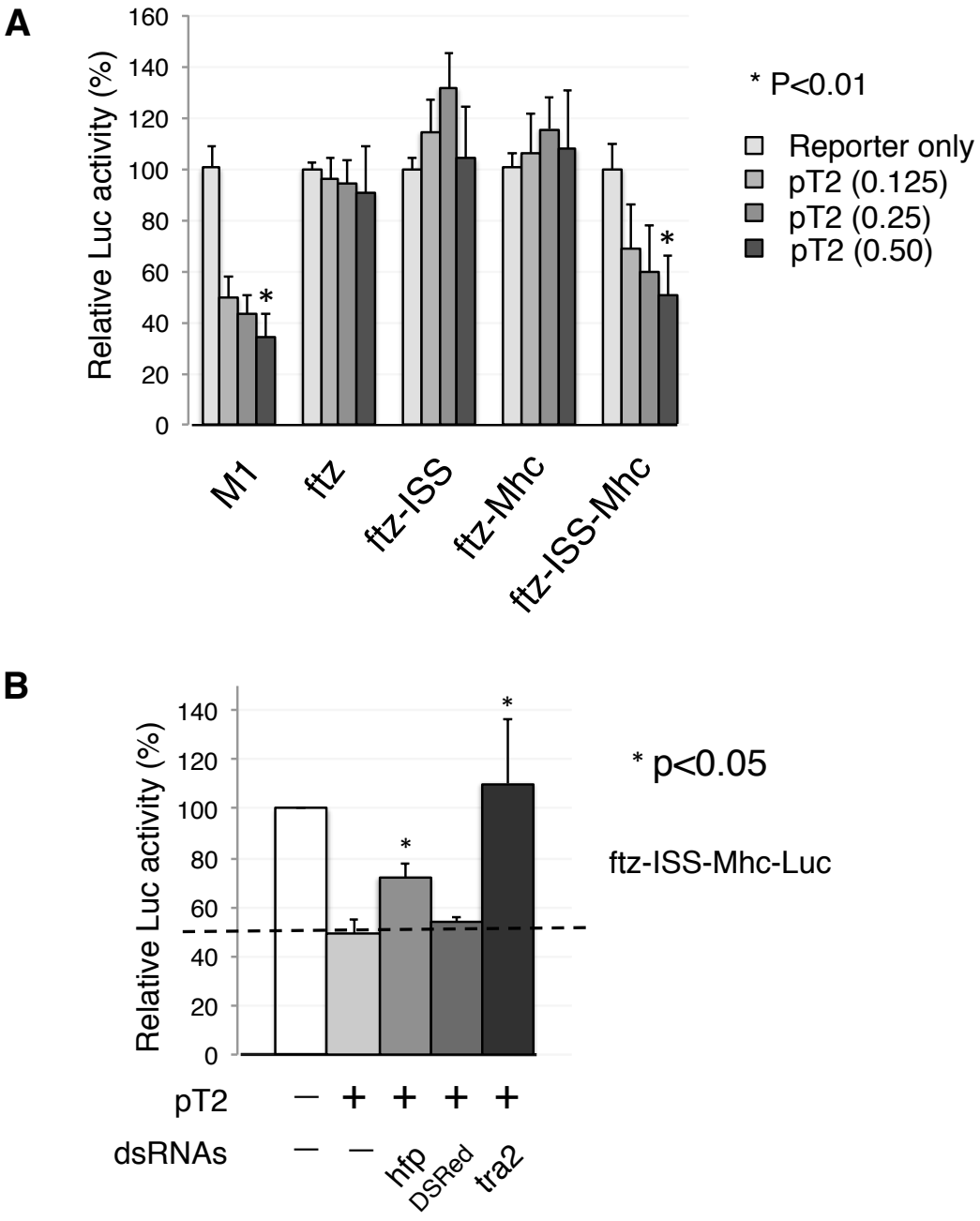


Figure 3-4. Diagrams of luciferase reporters based on ftz gene backbone

The boxes represent exons and horizontal lines represent introns. "Luc" is the luciferase cDNA fused in frame with upstream coding sequence. The M1 intron contains a weak 3' splice site, while ftz intron has a strong one. The strong 3' splice site of ftz intron was replaced with another weak 3' splice site from Mhc intron 5 in the reporters of ftz-Mhc-Luc and ftz-ISS-Mhc-Luc. ISS element is indicated with a thickened line within M1 intron. It was inserted into the introns of ftz-ISS-Luc and ftz-ISS-Mhc-Luc. Intronic sequences close to the 3' splice sites are listed below each diagram with red color and italic.

To test which elements are sufficient for the M1 repression mediated by Hfp and Tra2, the luciferase activities from different reporters were measured. As shown in figure 3-5, only when both ISS and a weak 3′ splice site were introduced into the ftz intron, the reporter (ftz-ISS-Mhc) underwent dose-dependent repression by Tra2-PC similarly to the M1 reporter. However ftz-based reporters with only the ISS or the mhc 3′ splice site failed to show repression in response to elevated Tra2-PC. This confirms that both the ISS and a weak 3′ splice site are required for the repression mediated by Hfp and Tra2. Further we verified that the splicing repression of ftz-ISS-mhc reporter was reversed by the knockdown of Hfp and Tra2 in a similar manner as found with the M1 reporter. But no effect was seen with the treatment of the control dsRNA (figure 3-5). Moreover the knockdown of Hfp led to a similar reversal of both M1 reporter and ftz-ISS-Mhc reporter when tested in the presence of only endogenous Tra2 (figure 3-5). To test the possibility that endogenous Hfp is present in sufficient levels to drive maximal M1 repression, a small amount of Tra2-PC, sufficient to cause a small increase in the M1 repression, was cotransfected with increasing amounts of the 68 kD Half pint isoform (Hfp68) (figure 3-6). The increasing amounts of Hfp68 did not produce further repression of M1 splicing. This indicates that endogenous Hfp68 levels do not limit the degree of M1 repression observed. Taken together the above findings indicate that the repressive function of Hfp on splicing is specific to some introns that contain certain critical elements. In this case, both ISS and a weak 3′ splice site are necessary for the splicing repression of M1 intron by Hfp and Tra2.


A
Relative Luc activity (%)
160
140
120
100
80
60
40
20
0
*
M1
ftz
ftz-ISS
ftz-Mhc
ftz-ISS-Mhc
* P<0.01
Reporter only
pT2 (0.125)
pT2 (0.25)
pT2 (0.50)
B
Relative Luc activity (%)
140
120
100
80
60
40
20
0
*
*
* p<0.05
ftz-ISS-Mhc-Luc
pT2 – + + + +
dsRNAs – – hfp DSRed tra2

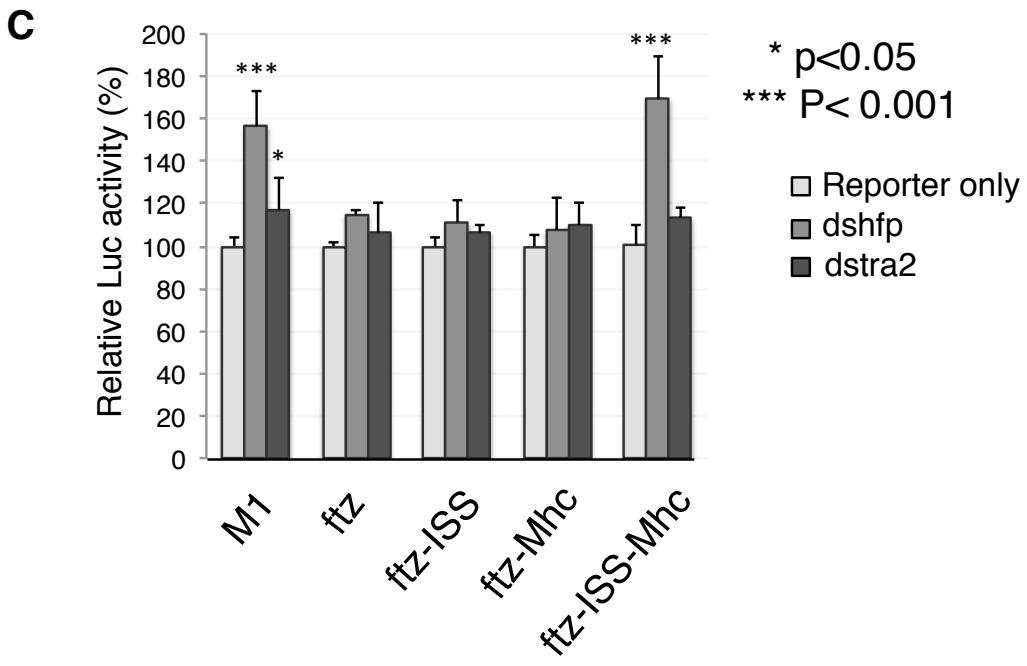


Figure 3-5. Both ISS and a weak 3′ splice site are required for the splicing repression by Tra2 and Hfp

(A) S2 cells were transfected with reporters and different amounts of pTra2. The effects of Tra2 on the luciferase activities are shown in the graph. Percent luciferase activity is in relation to that of the reporter only. Results with significant change are indicated (*). Only both ISS and weak 3′ splice site present in the intron have the similar effect with that of M1 reporter. (B) The knockdown of endogenous Hfp and transfected Tra2 level restored the luciferase activities and diminished splicing repression of M1 intron. No effect was observed when treated with the control dsRNA. (C) Reporters were transfected into S2 cells and treated with both Hfp dsRNA and tra2 dsRNA. Percent luciferase activity in relation to that of untreated group is shown in the graph. Significant

change is indicated (*). Only ftz-ISS-Mhc reporter has the similar response to the dshfp treatment as the M1 reporter.

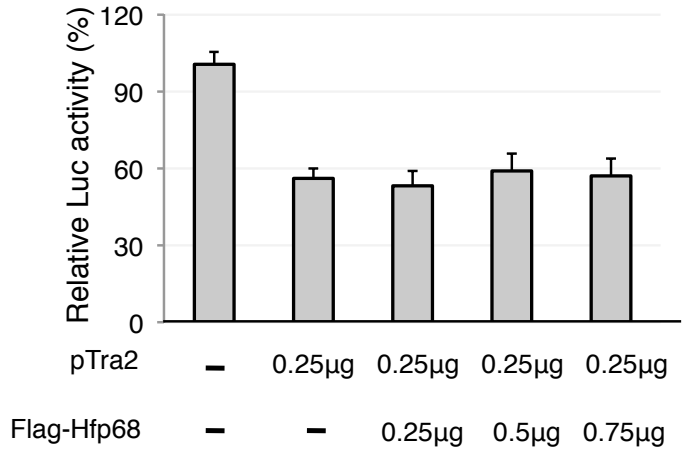


Figure 3-6. Increasing amounts of Hfp68 cannot repress M1 intron splicing

The luciferase assay carried out after cotransfection with pTra2 and different amounts of Flag-Hfp68. Repression by endogenous Hfp and transfected Tra2-PC was not augmented by increasing the Hfp68 level.

4. Half pint antagonizes U2AF50 activity on M1 intron

Initially the binding and functional activity of PUF60, the mammalian homologue of Hfp, was found to be at the 3′ splice site of a few introns tested. PUF60 could recognize a relatively weak polypyrimidine tract that is different from canonical ones recognized by U2AF65. Our reporter experiments have suggested that Drosophila Hfp also represses splicing through the 3′ region of M1 intron. To test its relationship with U2AF50 in M1 intron, the effects of endogenous Hfp and U2AF50 on M1 splicing were measured with M1 reporter in S2 cells. The knockdown of Hfp increased the luciferase activity from the M1 splicing reporter by two fold over that observed without dsRNA (figure 3-7) which is consistent with our previous results. However a parallel knockdown of U2AF50 resulted in a 2-fold reduction in the activity. Using a splicing reporter of the ftz intron, no effect was observed when endogenous Hfp was decreased, but the luciferase activity was again reduced after the knockdown by U2AF50 dsRNA. These results suggest that U2AF50 and Hfp have opposite roles in the regulation of M1 intron splicing.

Considering both U2AF50 and Hfp are splicing factors associating with the polypyrimidine tract, it is possible that these two factors compete with each other in this region. To test this idea, recombinant Hfp and U2AF50 were expressed and purified from bacteria. As shown in a gel shift assay (figure 3-8), U2AF50 could directly bind to an M1-derived RNA fragment containing both the polypyrimidine tract and the 3′SS, but Hfp cannot directly bind by itself under this in vitro conditions. Tested with longer RNA

substrates, recombinant Hfp only showed weak binding activity with the full-length M1 intron (figure 3-9). These results suggest that the recombinant Hfp protein probably requires other proteins to bind to the M1 intron efficiently.

However when Hfp was incubated together with U2AF50 and M1 3′ splice site, U2AF50 binding signal was decreased in respond to the increasing Hfp concentrations (figure 3-8). However no extra Hfp-associated signal was observed in these cases, which is consistent with previous observation that Hfp itself has a very low affinity with the elements in M1 intron. These in vitro binding assays are consistent with the findings from splicing reporter assays that suggest Hfp antagonizes the effect of U2AF50 in the 3′ part of M1 intron.

To test whether any other factors could help Hfp bind M1 transcript, recombinant Hfp was incubated with RNA fragments in S2 nuclear extract. The gel shift assays showed similar decreased signal of mobility shifted complex with relatively short RNA elements in response to elevated recombinant Hfp. However recombinant Hfp promoted a more stable protein-RNA complex when incubated with the full-length M1 intron in S2 nuclear extract (figure 3-10). That suggests that the stable association of Hfp and M1 intron need other sequences within the intron, and other factors within the S2 nuclear extract might associate with Hfp to form a more stable complex within the M1 intron.

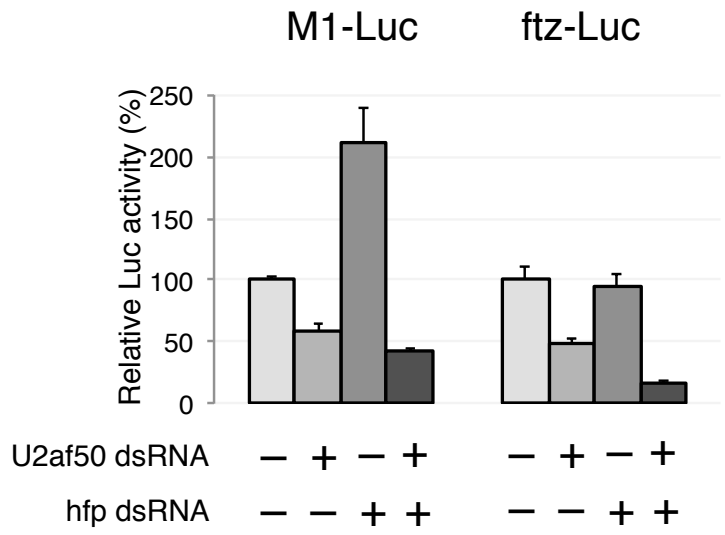


Figure 3-7. Hfp and U2AF50 have opposite effects on M1 intron splicing

S2 cells were transfected with M1 reporter or ftz reporter. Endogenous hfp and U2AF50 were knocked down by their dsRNAs. Percent luciferase activity in relation to untreated group is shown in the graph. Decreased hfp level had no effect on the luciferase activity of ftz reporter but caused significant increase of luciferase signal from M1 reporter. Decreasing U2AF50 level reduced intron splicing in both reporters.

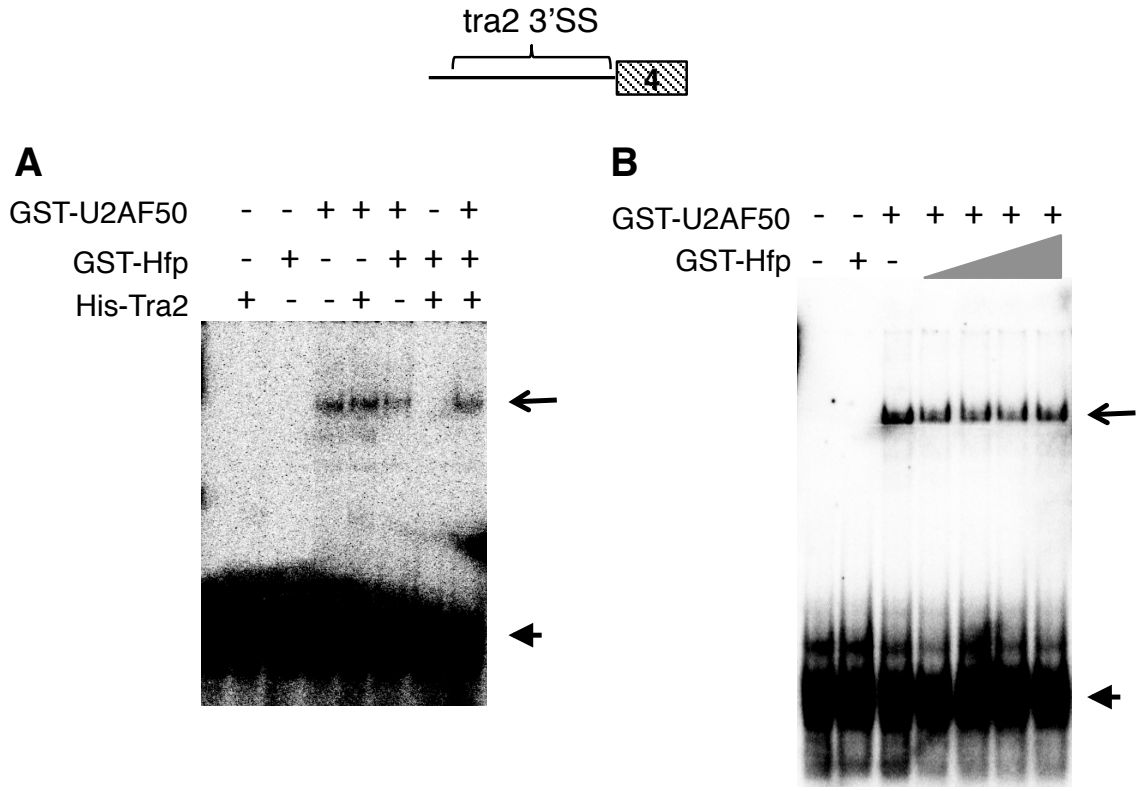


Figure 3-8. Hfp reduced binding affinity of U2AF50 with tra2 RNAs

(A) U2AF50 and Hfp protein were expressed with GST fusion tag and purified from bacteria. Tra2 protein was expressed with His tag and purified from baculovirus. Proteins are incubated together with the 3′ region of tra2 RNA in the binding buffer. The binding complexes were separated by the gel shift assay. RNA-protein complex and free RNA are indicated by the arrows and arrowheads. Hfp fusion protein reduces the amount of U2AF50 associating with RNA target while Tra2 protein does not. (B) Increasing Hfp amount greatly reduced the association of U2AF50 with tra2 3′ region. Triangle represent increased Hfp amount within the binding system.

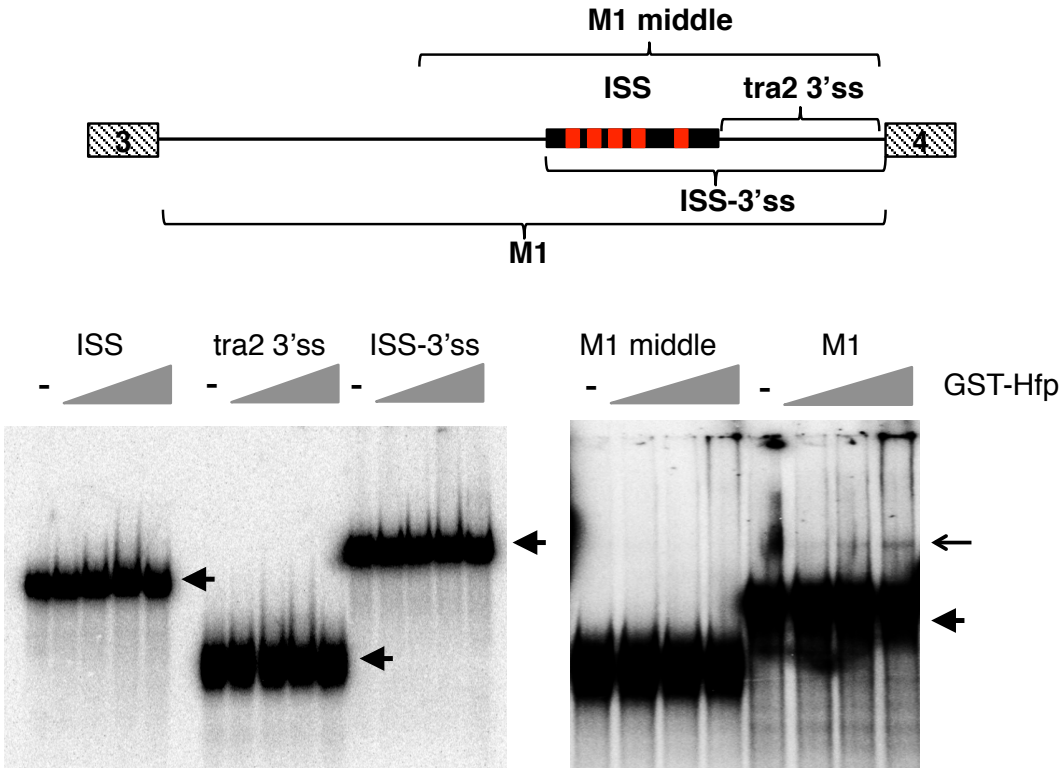


Figure 3-9. Hfp protein has a higher affinity with the full-length M1 intron

GST-Hfp was incubated with different lengths of M1 intron fragments in in-vitro binding conditions. RNA-protein complex was separated with gel shift assay. The fragments were indicated in the diagram above the gel images. Hfp-RNA complexes are indicated by the arrows and free RNA is labeled by the arrowheads.

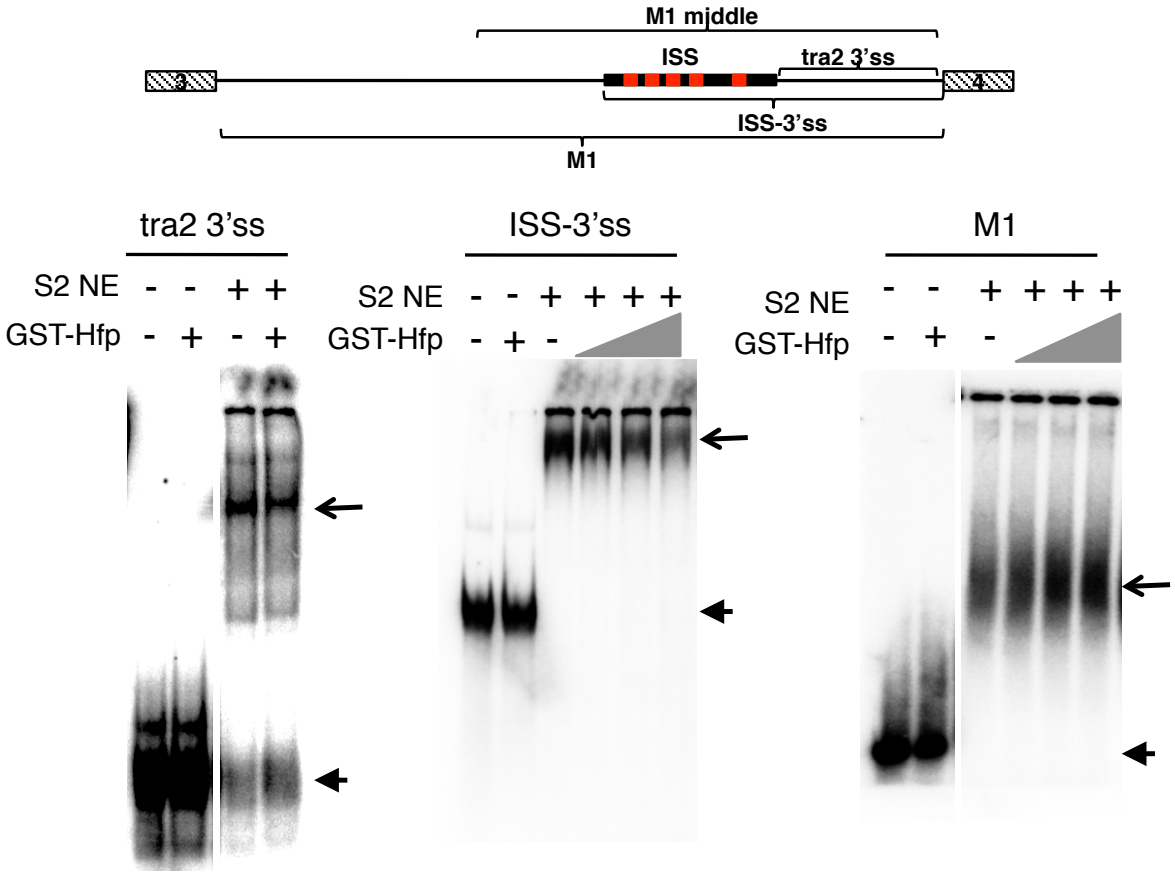


Figure 3-10. Hfp disrupted complex formed on short tra2 RNA fragments

GST-Hfp was incubated with S2 nuclear extracts and different lengths of tra2 RNA substrates. RNA-protein complexes were separated with the gel shift assay. Increasing Hfp amount decreased complex signal on both RNA substrates of tra2 3'ss and ISS-3'ss. When incubated with the whole M1 intron, the signal of the complex that S2 nuclear extract formed on the RNA fragment significantly increased in a hfp dose-dependent manner.

5. Half pint is required in vivo for the Tra2-dependent repression of M1 intron splicing but not for doublesex splicing

The splicing repression of M1 intron is most prominently observed in the male germline where about 50% of steady state RNA contains the M1 intron (Mattox and Baker, 1991) and the repression of splicing is entirely dependent on the presence of Tra2 protein. To determine if Hfp is also required for M1 repression in vivo we dissected testes from wild type and half pint mutant adults. Because strong loss of Hfp function results in lethality at earlier stages, this analysis was carried out with hypomorphic hfp mutations that allow adult survival (Van Buskirk and Schupbach, 2002). As shown in Figure 3-11 endogenous M1 splicing efficiency was significantly increased when half pint function was reduced by these mutations. Similar effect was seen in different genetic background of hfp mutants. Also dramatic decrease of M1-containing mRNAs was seen in the Tra2 mutant. These results demonstrate that Hfp is required for the repression of M1 splicing in vivo.

Tra2 is also known to activate alternative splice sites when it is bound to exonic splicing enhancers. To determine if Hfp is generally required for this activation function we tested whether female-specific splicing of *dsx* pre-mRNA is affected in Hfp mutant adult flies. RT-PCR was carried out on total RNAs with the primers that detect both the male and female specific *dsx* transcripts as diagrammed in Figure 3-11. The results of this experiment, shown in Figure 3-11 indicate that neither of two different Hfp loss-of-function genotypes reduced the selection of the female specific splice site that depends on the activation by Tra2. This suggests

that Hfp is not generally required for all Tra2-dependent alternative splicing events.

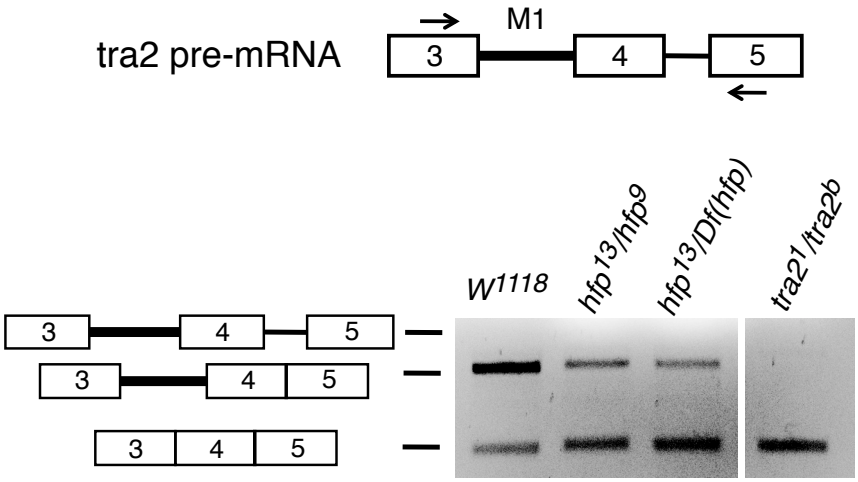


Figure 3-11. Hfp is required for M1 retention in vivo

RNA prepared from testis of wild type, Hfp mutant and Tra2 mutant flies were used to do RT-PCR. Primer positions are indicated in the diagram. Splicing products are shown on the left of the figure. In wild type testis, M1 retained mRNA is the major product, while in Hfp hypomorphic mutants, the retention level of M1 intron is dramatically decreased, which is similar with the effect seen in the tra2 mutant. Df(hfp)=Df(3L)Ar14-8.

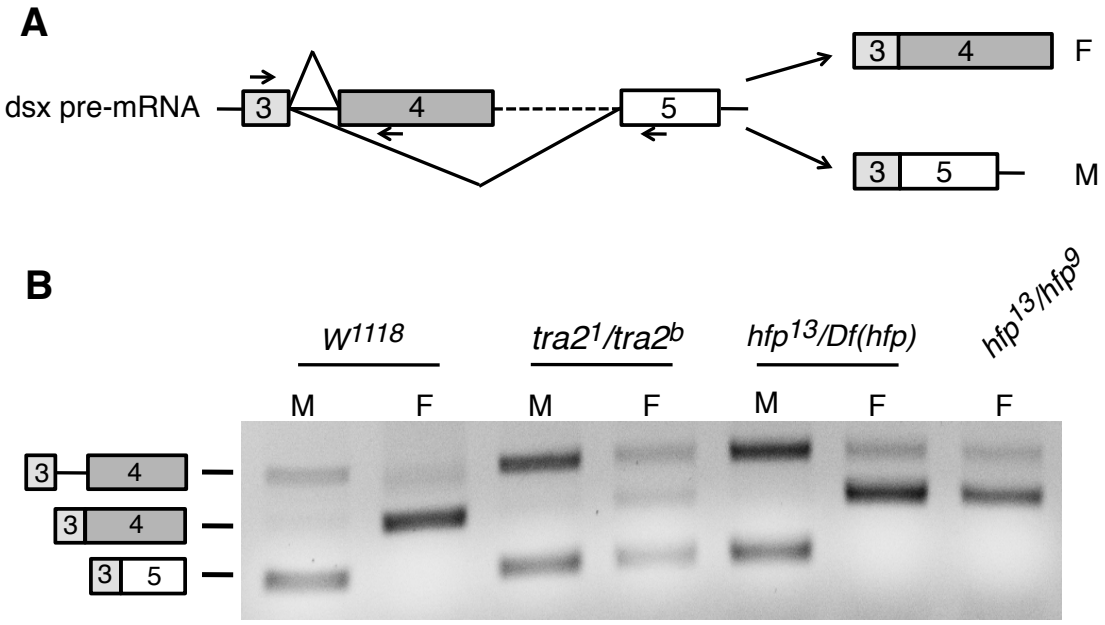


Figure 3-12. Hfp has no effect on the alternative splicing of doublesex pre-mRNA

(A) The alternative splicing of doublesex pre-mRNA is shown in the diagram. Three primers were used to amplify both male and female products of dsx splicing products and indicated in the diagram. (B) Whole fly RNA samples from different genetic background were prepared and used in RT-PCR assay. Compared with the Tra2 mutant, no male-specific splicing product was observed in Hfp female mutants.

6. The N-terminus of Hfp is required for its negative regulatory function

Analysis of the Drosophila transcriptome indicate that it contains as many as nine alternatively spliced mRNAs from the hfp gene (McQuilton et al., 2012)(figure 3-12). These mRNAs are predicted to encode either of two protein forms that differ by the presence of a 92 amino acid N-terminal sequence. Type A transcripts encode a 68 kDa protein (Hfp68) predicted to initiate translation in the second exon and consistent with the major Hfp product previously reported (Van Buskirk and Schupbach, 2002). All other Hfp transcripts contain in frame stop codons a short distance downstream of this initiation site. However initiation at a downstream start codon found in these transcripts could potentially produce a 58 kDa protein.

To study whether these two isoforms differ in their ability to repress M1 splicing, Hfp isoforms were introduced into S2 cells with M1 reporter and Tra2-PC. Endogenous Hfp was knocked down by a dsRNA specifically targeting the 3'UTR of endogenous Hfp (hfp3'UTR dsRNA). Introduced Hfp isoforms escaped the degradation brought by the hfp 3'UTR dsRNA since their cDNAs were inserted into the pSK backbone which contains a distinct SV40 3'UTR sequence. As shown in figure 3-13, endogenous Hfp was dramatically decreased by the treatment of hfp3'UTR dsRNA (lane 1 and 2), and the luciferase signal correspondingly increased to 150% of the level in the control with no dsRNA treatment. Overexpression of Hfp58 (lane 3 and 4 in figure 3-13) was not able to repress luciferase activity,

but introducing Hfp68 (lane 5 and 6 figure 3-13) returned the repression to the similar level of that with no Hfp knockdown. This phenomenon is also verified by the quantitative RT-PCR experiments on mRNAs from S2 cells treated in the same way (figure 3-14). The spliced/unspliced ratio of M1 reporter transcripts was observed to significantly increase with Hfp knockdown and to be restored after the expression of Hfp68 but not Hfp58. These results indicate that only Hfp68 is able to repress M1 intron splicing in S2 cells. Since the only difference between Hfp68 and Hfp58 lies in the N terminus, this suggests that the N terminus is critical for Hfp to function in the repression of M1 intron splicing.

Another interesting part for the Hfp protein is the UHM domain in the C terminus. Since it is thought to be involved in protein-protein interactions critical for splicing, we tested its potential role in the M1 retention. A deletion of the entire UHM from Hfp68 (HfpΔUHM) was made and expressed in S2 cells in the above rescue assay. As shown in Figure 3-15, overexpressing HfpΔUHM can strongly repress the restored luciferase signals by hfp 3'UTR dsRNA, as effectively as did introduced Hfp68. It suggests that the M1 repression by Hfp is likely independent of UHM domain even though it is thought to mediate the interactions between splicing factors.

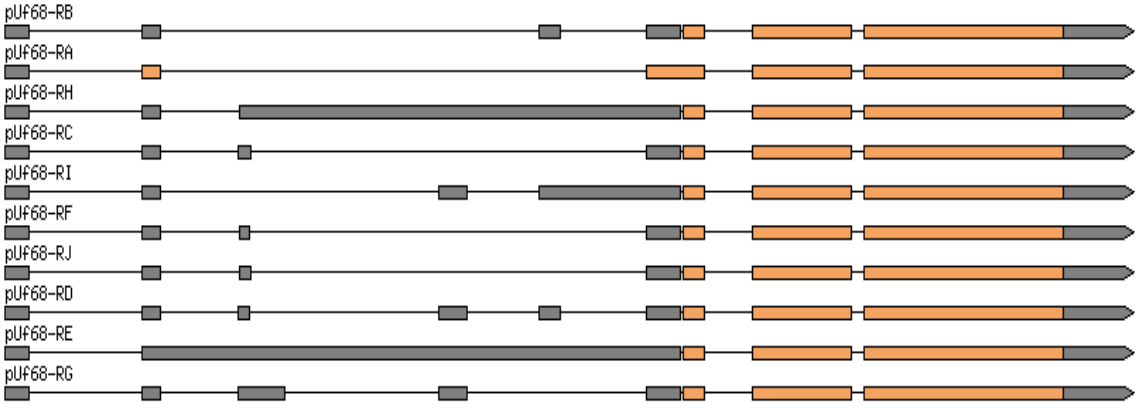
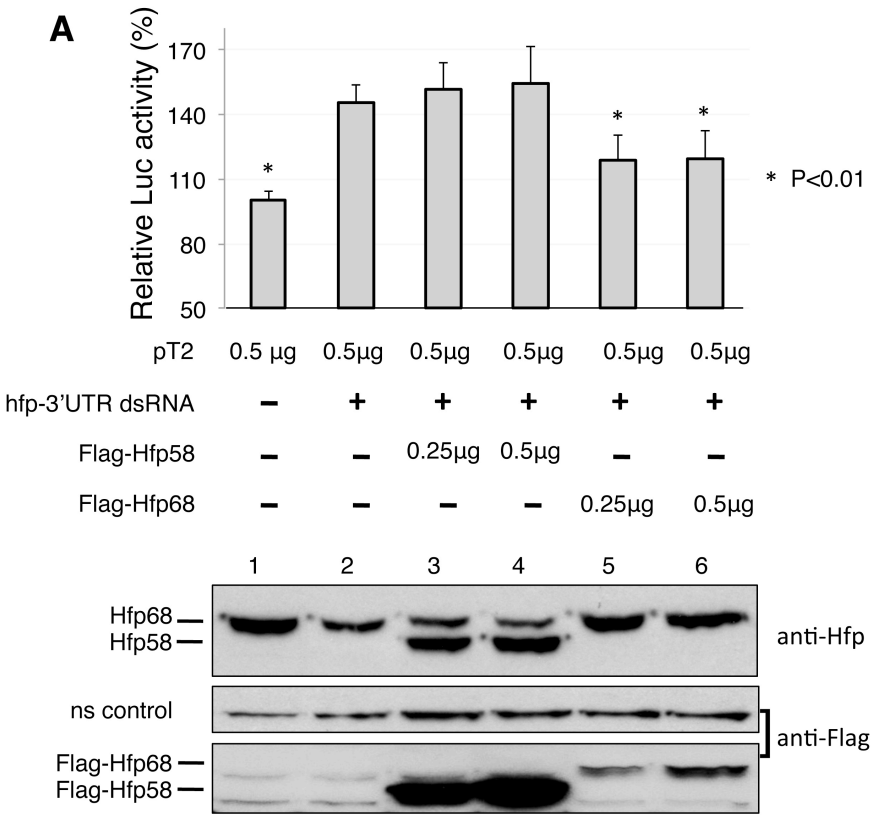


Figure 3-13. Diagram of Hfp mRNA isoforms

The exon-intron patterns giving rise to various Hfp encoding mRNAs as reported in GBrowse feature of flybase.org. The boxes correspond to exons, lines to introns. The gray shaded regions are predicted to be noncoding parts, the orange regions are predicted to be protein coding. Note that only the RA transcript has the potential to encode the Hfp68 isoform, all other transcripts are predicted to encode Hfp58. The transcription direction is indicated by the arrow.


A
Relative Luc activity (%)
170
140
110
80
50
* P<0.01
pT2
0.5 µg
0.5µg
0.5µg
0.5µg
0.5µg
0.5µg
hfp-3'UTR dsRNA
Flag-Hfp58
0.25µg
0.5µg
Flag-Hfp68
0.25µg
0.5µg
1
2
3
4
5
6
Hfp68
Hfp58
anti-Hfp
ns control
anti-Flag
Flag-Hfp68
Flag-Hfp58

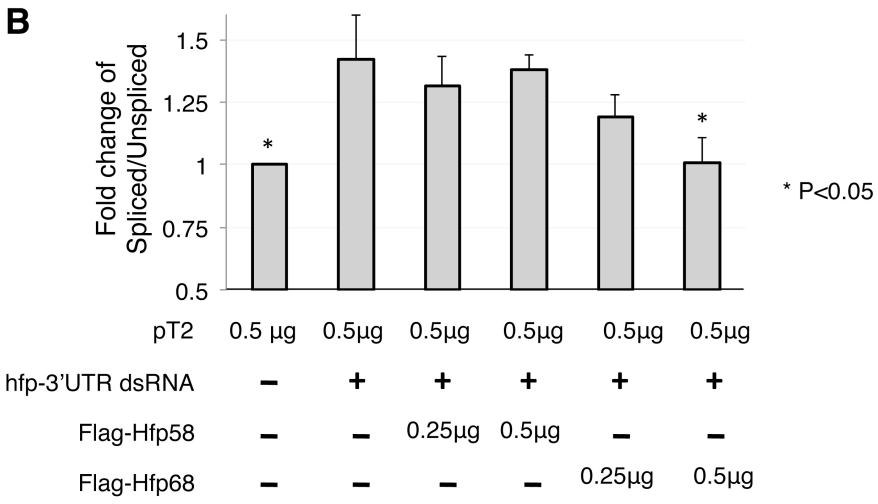

B
Fold change of Spliced/Unspliced
1.5
1.25
1
0.75
0.5
* P<0.05
pT2
0.5 µg
0.5µg
0.5µg
0.5µg
0.5µg
0.5µg
hfp-3'UTR dsRNA
Flag-Hfp58
0.25µg
0.5µg
Flag-Hfp68
0.25µg
0.5µg

Figure 3-14. Hfp58 cannot repress M1 splicing in S2 cells

(A) S2 cells were transfected with the M1 reporter and constructs expressing Flag-Hfp58 or Flag-Hfp68. Endogenous Hfp was knocked down by using a dsRNA targeted at its 3'UTR (hfp 3'UTR dsRNA). The ability of each Hfp isoform to restore repression was tested by the transfections with expression constructs containing the 3' UTR sequence from SV40 that are not affected by the dsRNA. Expression levels of Hfp58 and Hfp68 were detected by western blot shown below the chart. The 92 kD band shown is a non-specific cross-reacting protein from the same gel that is used to represent loading. The effect on M1 splicing was measured by luciferase assay. (B) Quantitative RT-PCR analysis of total cellular RNA from cells treated as in (A). The ratio of spliced/unspliced M1 reporter transcripts was determined using the primers as shown in the diagram. Significant change compared with the control treated with hfp 3'UTR dsRNA only is indicated (*).

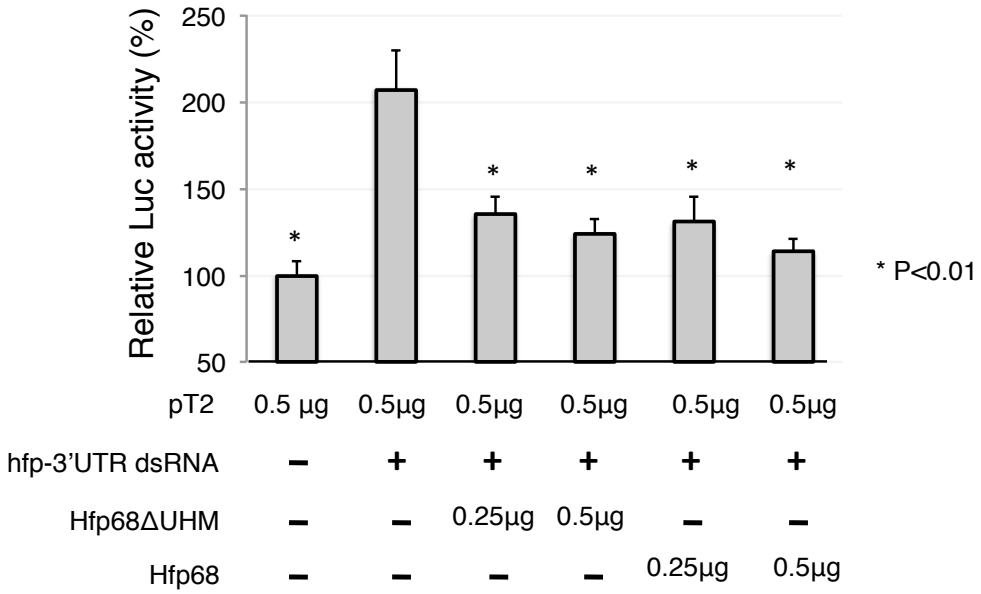


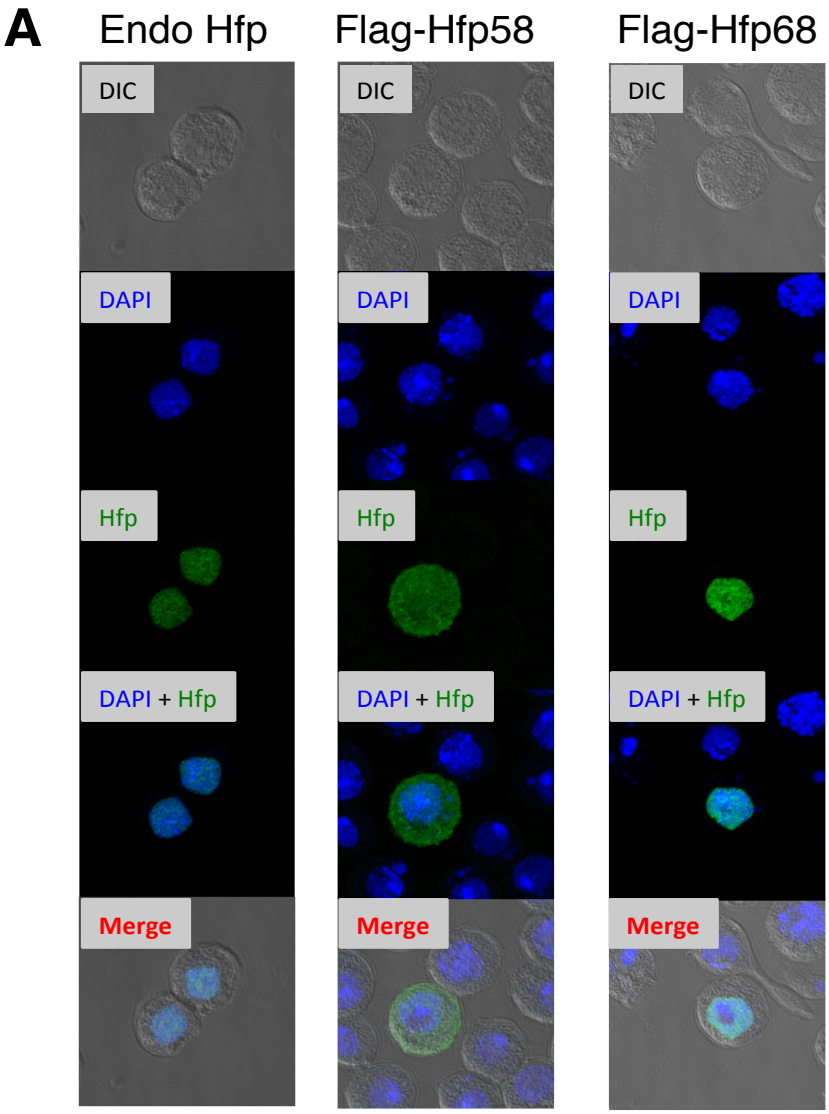
Figure 3-15. The UHM domain of Hfp has no effect on the regulation of M1 splicing

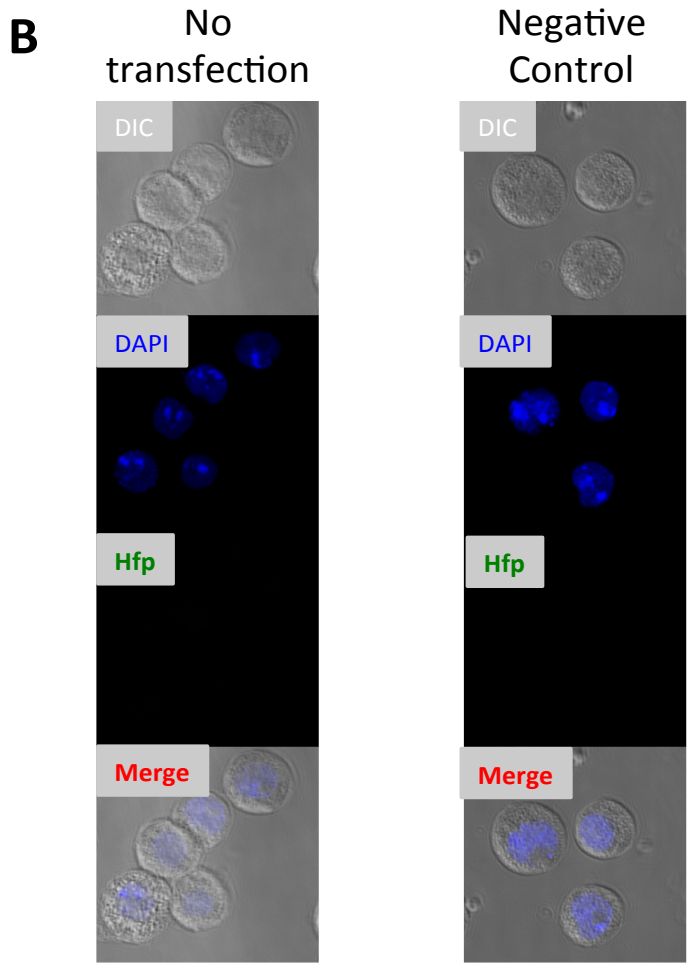
The role of the conserved C-terminal UHM domain in the splicing repression was examined using the same approach as in figure 3-14. A construct in which the UHM domain was deleted from Hfp68 (HfpΔUHM) was tested for its ability to restore the repression and found to be nearly identical to the full-length Hfp68. Results that affected significantly from the control treated with hfp-3'UTR dsRNA only are indicated (*).

7. The Hfp58 isoform that lacks N terminus has different subcellular localization with full-length Hfp68

To investigate why Hfp58 differs in its function from Hfp68, we tested if it has similar access to the pre-mRNA in cells. Subcellular localization of both Hfp isoforms were studied in S2 cells by immunostaining with antibodies to both Hfp and the Flag epitope tag. As shown in figure 3-16, transfected Flag-Hfp68 is mainly present in the nucleus as indicated by coincidence with DAPI signal. The same pattern is seen in the staining of endogenous Hfp with anti-hfp antibody in S2 cells where Hfp68 is the primary isoform. However Flag-Hfp58 signal was found to divided almost equally between the nucleus and cytoplasm. These results showed distinct subcellular localization of hfp isoforms in S2 cells that is likely to contribute to the observed difference of their functions in the regulation of M1 splicing.

To quantitate the distribution of these two isoforms we analyzed multiple confocal images. Green signal representing Hfp isoforms were collected within both the nucleus and the whole cells by selecting the 3-dimension space. The percent of Hfp signal in the nucleus in related to that in the whole cell was summarized in the graph (figure 3-16 C). Nearly half of the Hfp58 was located within nucleus while almost all of Hfp68 was contained in nucleus. These results showed distinct subcellular localization of hfp isoforms in S2 cells that is likely to contribute to the observed difference of their functions in the regulation of M1 splicing.


A
Endo Hfp
Flag-Hfp58
Flag-Hfp68
DIC
DAPI
Hfp
DAPI + Hfp
Merge


B
No transfection
Negative Control
DIC
DAPI
Hfp
Merge
DIC
DAPI
Hfp
Merge

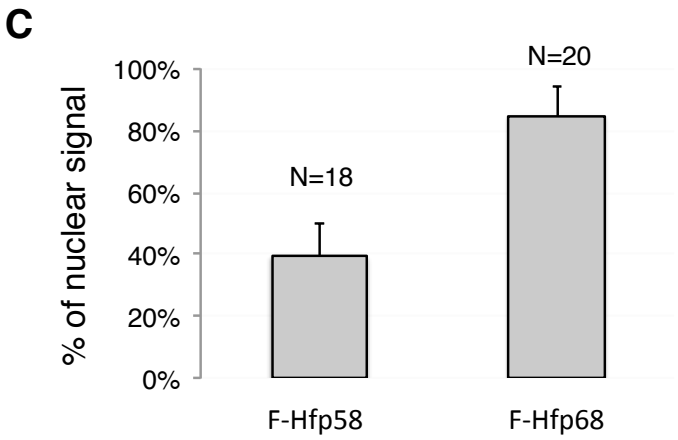

C
% of nuclear signal
100%
80%
60%
40%
20%
0%
N=18
N=20
F-Hfp58
F-Hfp68

Figure 3-16. Hfp isoforms have different subcellular localization in S2 cells

(A) Endogenous Hfp localization (Endo Hfp) detected with anti-hfp antibody against its C terminal region is shown in comparison to the localization of Flag-Hfp58 and Flag-Hfp68 in cells transfected with constructs expressing each form. Anti-Flag antibody was used to stain transfected Hfp protein. Both Hfp (green) and DAPI immunfluoescent staining (blue) are overlaid on DIC images of the same cells in the bottom row. (B) "No transfection" is the control that both anti-flag antibody and the secondary antibody were used to stain S2 cells with no transfection performed. "Negative control" is the control that only the secondary antibody was used to stain S2 cells with no transfection performed. (C) The subcellular localizations of Hfp isoforms were analyzed by image analysis. Flag-Hfp68 and Flag-Hfp58 were transfected into S2 cells separately. The percent of signal detected within the nucleus as determined by Imaris 7.3, is shown for both Flag-Hfp58 and Flag-Hfp68.

8. Half pint has two isoforms in Drosophila and the expression pattern shows sexual difference

Even though we found two Hfp isoforms differed in their behaviors on both the subcellular localization and the ability to repress M1 splicing in S2 cells, it is still unclear whether they are both naturally expressed in vivo. We tested this in Drosophila and in S2 cells by western blot with a monoclonal anti-hfp antibody that recognizes an epitope in the C-terminus of both Hfp isoforms. Figure 3-17 shows that two bands were detected in wild type flies and both are reduced in Hfp deficient mutant. Also both bands are increased when corresponding cDNAs of hfp isoforms were introduced into S2 cells (figure 3-17). In the overexposed western blot film from the experiment in S2 cells (figure 3-17 C), Hfp58 could be observed and its level was reduced by the treatment of Hfp dsRNA.

More interestingly, Hfp68 is the major isoform in male flies, but abundant Hfp58 was observed in female flies (figure 3-18). When exploring their expression pattern in dissected testis and ovary, Hfp68 was found to be the primary isoform in testis while the major isoform in ovary is Hfp58. Taken together, these observations indicate that both isoforms exist in Drosophila and their expression patterns differs between males and females.

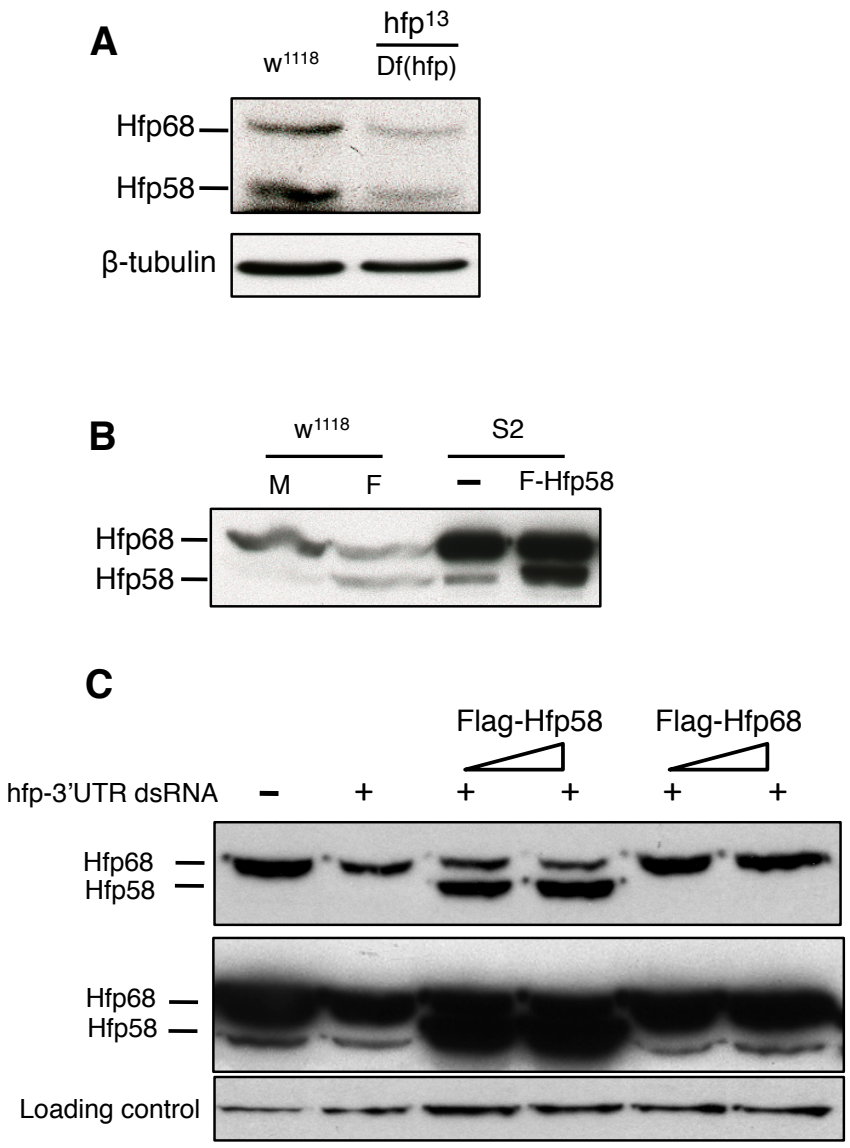

A
w1118
hfp13
Df(hfp)
Hfp68
Hfp58
β-tubulin
B
w1118
S2
M
F
–
F-Hfp58
Hfp68
Hfp58
C
Flag-Hfp58
Flag-Hfp68
hfp-3'UTR dsRNA
–
+
+
+
+
+
Hfp68
Hfp58
Hfp68
Hfp58
Loading control

Figure 3-17. Two Hfp isoforms are expressed in vivo

(A) Western blot showing the comparison of Hfp expression in lysates from a mixture of Drosophila male and female adult flies with various genotypes is shown. Both forms are observed in w^{1118} adults (carrying two wild type alleles of hfp), but are both reduced in hfp13/Df(hfp). (B) Expression of Hfp68 and Hfp58 in w^{1118} male (M) and female (F) adult flies is shown as detected by western blot. For comparison lysates from S2 cells (S2) and S2 cells transfected with Flag-Hfp58 (F-Hfp58) were loaded on the same gel. (C) Both endogenous Hfp isoforms were knocked down by hfp 3'UTR dsRNA. The image of western blot from figure 3-14A was over exposed (the middle panel). Hfp58 could be seen under the Hfp68 signal. And Hfp58 signal showed reduced compared with lane 1 of no dsRNA treatment.

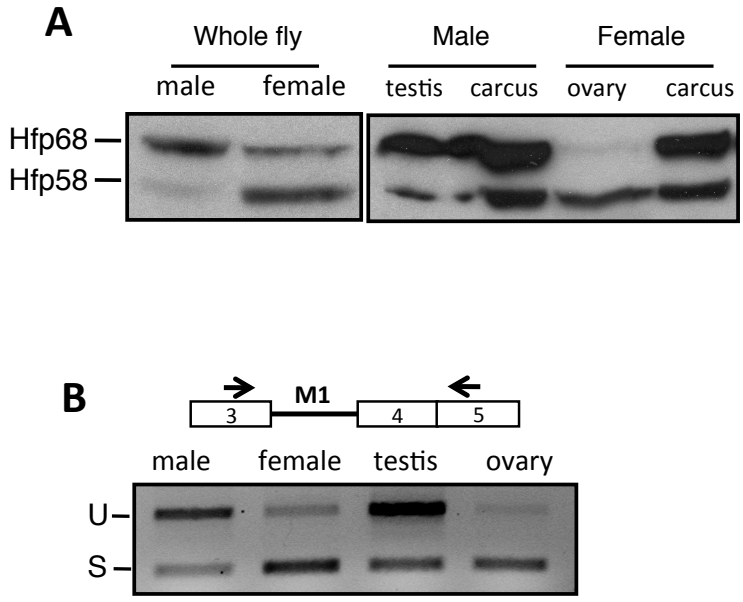


Figure 3-18. Sexual difference of the expression patterns of Hfp isoforms

(A) Western blot of Hfp isoforms in Drosophila with anti-Hfp antibody. Hfp58 is more abundant in female flies and also is the dominant form in oocytes compared with Hfp68 in testes. (B) RT-PCR of M1 intron splicing in Drosophila. M1 is mostly retained in testis compared with that in oocytes. Primer locations are indicated in the diagram above the image.

Discussion

RNA splicing is a complex process that many positive and negative regulators are involved to make a final splicing decision. As for SR proteins, they have been studied extensively for its role on how to promote pre-mRNA splicing. Here we use Drosophila Tra2 as a model to explore how an SR protein negatively regulates alternative splicing. The reporter system we used here is designed for RNAi screening in S2 cells. Since Tra2 represses M1 intron splicing in testis, it is reasonably to think that there are some testis specific factors responsible for the M1 retention happening. However, our previous result (Qi et al., 2006) clearly showed that Tra2 has the potential to repress M1 splicing in somatic cells as long as relatively high level of Tra2 was present. That is consistent with early observations from our lab that tra2 gene uses stronger promoter in male germ cells compared with the somatic promoter to get relatively high level of Tra2 and further achieve the M1 retention in testis(Mattox et al., 1996).

Hfp was known as a 3’ splice site regulator in mammalian system to promote weak 3’ splice site recognition (Valcarcel et al., 2007). However in our RNAi screening, it was identified as a co-repressor of Tra2 to achieve M1 intron retention. It is interesting that two splicing regulators who both were known to promote RNA splicing are also required for the repression of M1 intron splicing. Our previous data has shown that the intronic

splicing silencer was sufficient for Tra2 to repress intron splicing in vitro. However in S2 cells, we found only ISS itself cannot bring the M1 intron retained in the final mRNA but a weak 3′ splice site is also required at the same time, which is consistent with the observations from our previous in vivo study. When intrinsic M1 3′ splice site was changed into a consensus one, M1 retention was abolished in testis. When the 3′ splice site was replaced by another weak one, M1 retention was restored in a similar level (Chandler et al., 2001). Here we showed that the M1 repression by Hfp and Tra2 depends on both ISS and a weak 3′ splice site.

There are several studies suggest an exon definition model to explain the splicing repression by SR proteins. Our previous results also suggested that the M1 intron retention mediated by Tra2 could be the result of a transformation from an intron definition to an exon definition (Shen and Mattox, 2012). From our in vitro studies, it suggested that Hfp could compete with U2AF50's activity in the 3′ region of the M1 intron and form a more stable complex with Tra2 and other factors on the whole M1, further define the whole intron with flanking exons as a big exon and cause the intron retention. It will be interesting to test whether the complex formed on the M1 intron contain the components of complex E or complex A?

Hfp is originally identified as a transcriptional factor that negatively regulate c-myc expression. In our RNAi screen, it is

possible that the effect we saw by Hfp knockdown is from down-regulation of Tra2 level. However our western blot result indicates that Tra2 level was not affected. And the result of RNA immunoprecipitation assay further showed that Hfp could specifically associate with tra2 RNA. These observations lead to the conclusion that Hfp contributes to the repression of M1 splicing through the function as a splicing factor instead of a transcriptional factor.

Protein isoforms could have distinct biological functions in cellular processes (Cartegni et al., 2006; Markovtsov et al., 2000). Taking Tra2 as an example, its 179aa isoform was shown unable to repress M1 intron splicing in testis, but the other two isoforms 264aa and 226aa have the abilities to activate dsx female specific splicing in somatic cells and repress M1 splicing in male germline (Mattox et al., 1996). Based on the transcriptome annotation, the two Hfp protein isoforms are encoded by different mRNAs that depend on whether extra exon is included in the first intron. However no report has ever shown the presence of these two protein isoforms in vivo and any functional differences between them. In this study we first showed that both of the Hfp isoforms are expressed in S2 cells and Drosophila, even though S2 cell contains very low abundance of Hfp58. More interestingly, it is the full length Hfp68 instead of Hfp58 that can repress M1 splicing in our S2 cell system, which suggests that the N terminus of Hfp68, the only difference between the isoforms, is required for Hfp's negative regulatory function. Within the N terminus, there are four

serine and arginine (SR) dipeptides that are not present in mammalian homologue PUF60 (figure 3-19). It is possible that this RS containing part is responsible for the protein localization within the cell, even though it shows no similarity with any conserved nuclear-localization signal. Although some RS domains of SR proteins have shown the ability to locate protein into the nucleus (Caceres et al., 1998), the RS-containing N terminus of Hfp68 is not comparable with those RS domains with highly repeated RS dipeptides. So it is not clear how much role of the four RS dipeptides played on the nuclear localization of Hfp68. It will be very informative to see whether PUF60 could repress M1 intron splicing in flies, or Hfp68 with these RS dipeptides mutations could still help Tra2 achieve M1 intron retention.

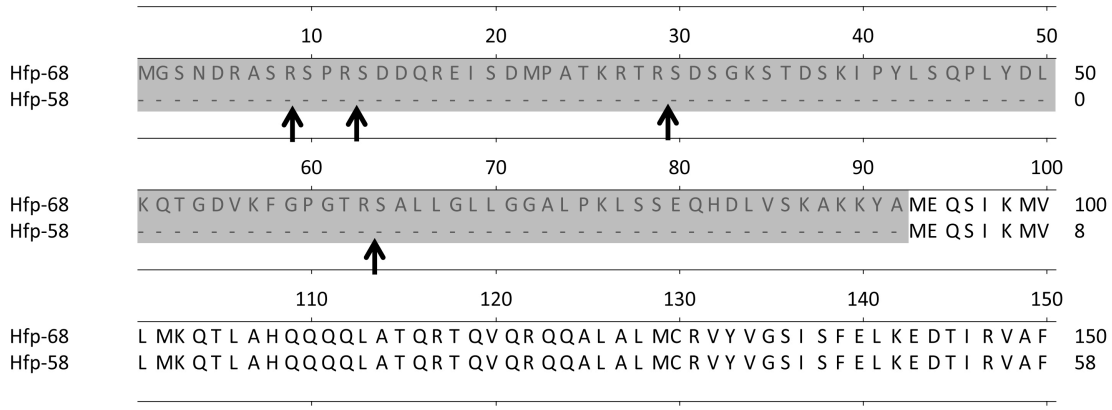


Figure 3-19. The alignment of protein sequences of Hfp68 and Hfp58

The protein sequences of Hfp isoforms were aligned by using MegAlign software. Only the N terminal parts are shown. The difference between the isoforms is marked with shaded color. Four Arginine-Serine dipeptides within the N terminus are indicated with the arrows.

Although this unconserved N-terminal domain seems required for M1 intron retention, the UHM domain located in the C terminus is not needed for this repression to happen. It suggests the interaction between U2AFs and U2AF-U2snRNP mediated by UHM domain, which is important for activating splicing, is not necessary for the splicing repression of M1 intron. This phenomenon also suggests that individual domains within Hfp68 are responsible for its different behaviors during alternative splicing.

The difference of subcellular localization between Hfp58 and Hfp68 was observed in S2 cells. It helps to explain their functional difference on M1 intron splicing. Splicing factors can regulate alternative splicing by adjusting their functional level accessible to the targets. Some SR proteins are well known in shuttling between nucleus and cytoplasm to regulate their nuclear concentration, and further regulate RNA splicing (Caceres et al., 1998; Cazalla et al., 2002). Hfp58 distributes evenly within the whole cell compared with Hfp68 highly enriched in nucleus. By this way, nuclear concentration of Hfp protein was decreased when endogenous Hfp68 was knocked down and Flag-Hfp58 was overexpressed. With low concentration of nuclear Hfp protein, its repressive function on M1 splicing was compromised, which is clearly shown in our luciferase and qRT-PCR assays. However, we still cannot exclude the possibility that Hfp58 has the ability to repress M1 intron splicing since there is still some within the nucleus. Other experiments need to answer whether this RS containing N terminus endows splicing activity to half pint protein in vivo. There is evidence showing the N terminus of PUF60 is sufficient to repress

c-myc transcription as a transcriptional factor, but our preliminary data showed the N terminus only of Drosophila Hfp cannot repress M1 splicing in S2 cells.

Another interesting question is if Hfp58 expresses in vivo and its localization is across the whole cell, what is its role in the cytoplasm? So far all of the functions of Hfp are restricted within nucleus, both transcription and RNA splicing. Instead of decreasing its level in nucleus to regulate RNA splicing as we speculated above, is there any possible function on translational regulation or protein degradation? Even no cytoplasmic function has been reported for Hfp, in vitro interaction studies identified some interacting proteins that function in cytoplasm might shed light for future directions.

The function division among protein isoforms can be seen extensively in vivo. As a bifunctional regulator with two isoforms, it will be very interesting to know whether Hfp isoforms have functional division in vivo that one isoform is responsible for transcriptional regulation while the other one participate in RNA splicing. Or both isoforms could be responsible for transcription and splicing regulation, but two isoforms exhibit different behaviors for particular substrates. For example as a splicing factor, one isoform is only for splicing activations and the other one is just for repressions. There is report showing that Hfp is differently spliced in male and female flies (Hartmann et al., 2011). Our preliminary data also suggested that these two isoforms

are differentially expressed in fly males and females. Considering M1 intron is only retained in testis while Tra2 level is similar in both testis and ovary, there could be some sex-specific functions performed by different Hfp isoforms. Although we found Hfp68 and Hfp58 have distinct subcellular localization, no data has ever shown the similar difference present in vivo.

To verify these functional roles in vivo, isoform specific mutated flies will be extremely helpful. However since one major function of Hfp resides in germ cells, there is technical difficulty to delete or overexpress hfp isoforms only in male germ cells. An improved gal4-UAS tool that could avoid this barrier is worth a try to test the ideas (Haley et al., 2010; Ni et al., 2011).

As a cell-based method to study RNA splicing, a potential drawback of the experiments done in S2 cells is the manner of transient transfection. With multiple reporters or vectors cotransfected and also treatment of dsRNAs, considering the efficiency and cell status of each time experiment, the luciferase activity could vary a lot from time to time. To avoid this kind of fluctuation, stable transfected cell lines should be suggested to establish for the future studies. The stable cell lines could contain both the M1 reporter and Tra2-PC cDNA.

CHAPTER FOUR

The Regulation of Alternative Splicing of Taf1 pre-mRNA by Half Pint and Tra2 in Drosophila Testis

Introduction

Alternative splicing, as an essential regulatory step of gene expression, also plays important roles in germline development. Numerous alternative splicing events have been reported during male germ line development as discussed in Chapter One. However the splicing factors that regulate these events are not known and how splicing regulator function is integrated into germ cell development is poorly understood.

The negative autoregulation of M1 splicing by Tra2 is known to be important for fertility in male flies. Transgenic flies carrying copies of the Tra2 gene in which autoregulation is impossible due to a deletion of the M1 intron were found to exhibit dose-dependent sterility (McGuffin et al., 1998). The sterile males were found to produce mature sperm, but these do not move into seminal vesicle indicating that they are immotile. Given that Tra2-PC is constitutively expressed from this transgene and is the only isoform of Tra2 that is genetically functional in the germline, these results suggest that sterility can result from excess Tra2 activity. Thus a limited level of Tra2 in germline is critical for

male fertility and negative autoregulation plays an important role in limiting Tra2 expression during spermatogenesis.

Since Half pint was identified in our RNAi screen as a Tra2 co-repressor of M1 splicing and has also been shown to affect alternative splicing in the female germ line (Van Buskirk and Schupbach, 2002), it is interesting to know whether Half pint functions together with Tra2 in a common pathway to regulate other targets important in spermatogenesis. Both Half pint and Tra2 were recently implicated in the regulation of alternative splicing of transcripts from the taf1 gene in response to DNA damage and ATR signaling in Drosophila cultured cells (Katzenberger et al., 2009).

Taf1 (TBP associated factor 1) is a transcription factor and a subunit of TFIID. Genetic studies suggest it plays an important role in cell proliferation and viability in Drosophila. Its pre-mRNA is spliced into mRNAs encoding four distinct protein isoforms. These mRNAs differ from each other by the inclusions of exon 12a and exon 13a (figure 4-1). It has been shown that Taf1-2 mRNA is highly enriched in the fly testis (Katzenberger et al., 2006). Interestingly, several genes encoding other germline specific paralogues of TFIID components have been identified in Drosophila and are known to share similar mutant phenotypes suggesting an important role in the entry into meiosis and for spermiogenesis (Hiller et al., 2004). These factors differ from Taf1-2 in that their tissue specific expression is determined transcriptionally rather than by alternative splicing. Taf1 null mutants are lethal,

but hypomorphic alleles are sterile in both female and male flies (Wassarman et al., 2000). It is reported that Taf1 proteins expressed in the testis co-localize with other TFIID components and is thought that Taf1 may be required for the integrity of testis TFIID complex (Metcalf and Wassarman, 2007).

In this Chapter I will explore the relationship of Half pint and Tra2 in promoting germline development and test how they impact germline specific alternative splicing of Taf1 in vivo.

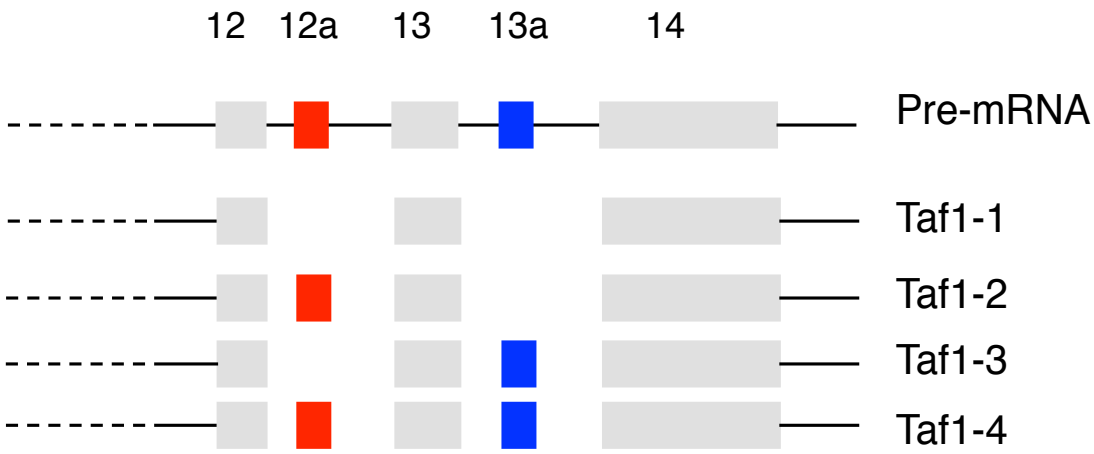


Figure 4-1. The diagram of taf1 mRNA isoforms

The exon-intron pattern of Taf1 pre-mRNA is shown. Gray boxes correspond to exons and horizontal lines to introns. Red boxes represent exon 12a and blue boxes represent exon 13a. Taf1 mRNA isoforms with different exon inclusion patterns are shown with the names labeled to the right side of the diagram.

Materials and Methods

Fly stocks

Stocks used bearing tra2 mutations had the following genotypes: *w^{1118}/B^sY; tra-2^B/CyO, w^{1118}/B^sY; tra-2^{PM6}/CyO, w^{1118}/B^sY; tra-2^{PM7}/CyO, w^{1118}/B^sY; B1 tra-2/CyO. w^{1118}/B^sY; Df(2R)Trix/CyO.*

Hfp mutants used were from the following strain genotypes and were obtained from Trudi Shupbach: *w;hfp^{13}/TM6B,Hu. w;hfp^{9}/TM6B,Hu. Df(3L)Ar14-8,red[1]/TM2,p(p). hfp^{3058},recl/TM3,Ser.*

Taf1 mutants were obtained from the Bloomington Drosophila stock center: *w^{1118};$taf1^{EP421}$/TM3,Sb. w^{1118};$taf1^{1}$ red^{1} e^{1}/TM3,Sb. y1; $taf1^{R14}$ red^{1} e^{1}/TM2.*

RNAi and GAL4 strains were of the following genotypes and were obtained from Drosophila Stock Center: *y sev;VAL20-Puf68(y^+v^+). y sev;VAL20-Taf1(y^+v^+). P[Gal4::VP16-nos.UTR]MVD1.*

The strain *w,P[m-w+,bam-Gal4-VP16]* was obtained from Dennis McKearin (Chen and McKearin, 2003).

Primers for PCR

Primers used in RT-PCR for Exon12a splicing were:

Exon 12 forward: 5′-GCATGCCTCCTCATCGAACTC

Exon 13 reverse: 5′-CATGCCATCCATGGCATCGG

Primers used in RT-PCR for Exon 13a splicing were:

Exon 13 forward: 5′-CCGATGCCATGGATGGCATGT

Exon 14 reverse: 5′-AGGCCCATTATCATCCTGCT

Primers used in qRT-PCR for Exon 12a splicing were:

Exon12a inclusion product: E1212a forward: 5′-CGTGGAGGAGGTCAAATCCC

E12a13 reverse: 5′-TCGTCGTCGTCCTCGTCATC

Exon12a skipping product: E1213 forward: 5′-CGTGGAGGAGGATCTCCAAT

E13 reverse: 5′-CGCCCTGATCTAAAATGCTC

Primers used in qRT-PCR for marker gene expression were:

Twine: forward 5′-ACAATTGGGAAGCCAGGGCGG,
reverse 5′-TCGTTGCTCCGCAGGTAGCG

fzo: forward 5′-TTGGGCTTCCGATCGCCGAG,
reverse 5′-AAAGGTGGCAGGGGCGAACA

aly: forward 5′-GGTCAGCAGTTTTCTGCACG
reverse 5′-AATCCGGAAGACTGAGCACG

can: forward 5′-GGAAATGTCATTGCGTCCCG
reverse 5′-GGGTCCTAATGGCTTCGTCG

bol: forward 5′-GTGAGCAAGGGCTACGGATT
reverse 5′-CTTTTTGATGGCCGGTGCAA

cycA: forward 5′-TTTTGAGCCAAATGGCGGTG
reverse 5′-TGGTGTAACTGTCGTCGGTG

cycB: forward 5′-GGCAGATCCGACAGATGGAG
reverse 5′-TGGACATCGTATGGTGCTCG

RpL32: forward 5′-AGCATACAGGCCCAAGATCG
reverse 5′-CTTCTTGAATCCGGTGGGCA

RT-PCR for genomic verification:

Hfp mutant: forward 5′-TCCCCACAGTTACTCAAAACCTATC
reverse 5′-GTTTGGTGCGAGTTAAAAAGTGTCT

Taf1ep421: forward 5′-CGGACATGCTAAGGGTTAATC

reverse 5′-CAGCATAGAATCCAGACCCA

Bam-gal4: forward 5′-AGGTGACCATAAATTGAAAC

reverse 5′-GTTCCAGTCTTTCTAGCCTT

Real-time PCR

cDNA was synthesized in 20μL of total volume with 1μg total RNA by Superscript first strand RNA kit. 2μL of cDNA from this reaction was used as template for PCR amplification. It was mixed with 2X Syber Green PCR mix from ABI and supplemented with primers at a concentration of 0.625 ng/μL. PCR was performed using the following cycle conditions: 50°C 2minutes and 95°C 10 minutes, followed by 95°C 15 seconds and 60°C 1 minute for 40 cycles.

Fertility test

Single male flies were cultured with three w^{1118} virgin females. Vials were cleared after flies were kept together for one week and offspring were counted at the pupal stage.

Live cyst dissection

Testis were dissected in the testis dissection buffer (TDB, 0.183M KCl, 47mM NaCl, 10mM Tris-Hcl, pH 6.8). Single testes were loaded on a slide with 30 μL TDB. The anterior third of the testis was peeled open and placed under a coverslip. Extra TDB was soaked gradually with Kimwipe under a phase contrast microscope (Leica DMR)

and observed until intact cysts were pushed out of the organ. Cells within intact cysts were counted. Pictures were taken with a Photometrics Quantix camera.

Testis immunostaining

Testis from wild type flies and other mutant flies were dissected in TDB and mounted onto slide in a 30µL (intact testes) or 20µl (squashed testes) volume of TDB and covered with a 20mm coverslip. Slides were frozen in liquid nitrogen for more than 30 minutes. The coverslip was then removed with a razor blade and immediately put into fixation buffer (4% formaldehyde, 8% acetic acid, 15% ethanol in 1XPBS). After incubating at room temperature for 10-15 minutes, slides were placed in 50% acetic acid buffer to wash for one minute, followed by three washes in PBS and then incubated for 30 minutes in 0.5% PBX (0.5% Triton X-100 in PBS). This was followed by blocking with 1% BSA in PBS for 30 minutes at room temperature followed and incubation with anti beta-tubulin antibody (1:200 in 1% BSA) (Developmental Studies Hybridoma Bank) at 4°C over night. The next day, slides were incubated with fluorescent secondary antibodies (diluted 1:500 in PBX) at room temperature for 2-2.5 hours after washing three times with PBX. Finally, they were stained with PI (1:5000) for 5 minutes at room temperature and washed three times. Images were taken with Nikon Eclipse Ti confocal microscope.

Single fly PCR

One fly was squashed with 200µL tip containing 50µL SB buffer (10mM Tris 8.0, 25mM NaCl, 1mM EDTA and 200ug proteinase K) in 1.5mL eppendorf tube. After macerating 20 times, residual SB buffer was expelled and the homogenate was incubated at 37°C for 30 minutes and then 95°C for 2 minutes. Samples were spun 7500 rpm for 10 minutes in an eppendorf microfuge. Finally 2µL of the supernatant was used as template for PCR reactions.

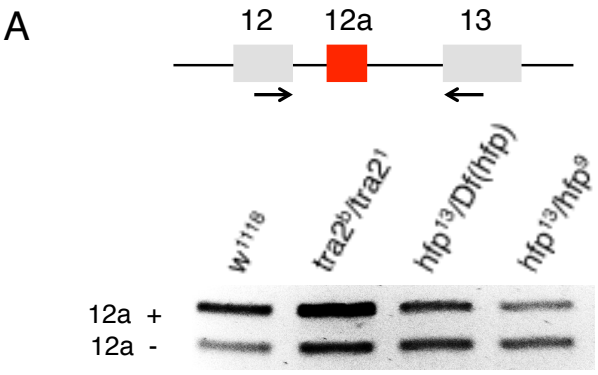
Results

1. Half pint and Tra2 have opposite effects on the alternative splicing of Taf1 exon 12a

Alternative splicing of Taf1 pre-mRNA produces four isoforms with different inclusion strategies of two alternative spliced exons: exon 12a and exon 13a. Previous studies conducted in S2 cells reported that in response to ATR signaling, Tra2 and Hfp were required for the upregulation of mRNAs encoding two Taf1 isoforms: Taf1-3, which includes exon 13a, and Taf1-4 which includes both exon 12a and exon 13a (Katzenberger et al., 2009). In vivo, the same authors found that Taf1 isoforms have distinct expression patterns in different tissues. Taf1-2 is highly enriched in the testis (Katzenberger et al., 2006). Exon 12a is the only alternatively spliced exon included in this mRNA. So I tested whether the splicing of this exon is changed in the testes of flies with loss of function mutations in tra2 and hfp. Testis RNA from several different heteroallelic mutant genotypes was isolated and used to do both conventional RT-PCR and as well as real time Q-PCR. As shown in figure 4-2, the RT-PCR result showed that more exon 12a was included in RNA from $tra2^{b}/tra2^{1}$ loss-of-function mutant testes, while it's skipping was increased over wild type controls in Hfp mutant testis. Confirming this observation, similar results were seen in Q-PCR experiments. Again, more exon 12a inclusion was observed in each of three different Tra2 mutant genotypes and less exon 12a inclusion was found in both hfp mutant genotypes tested. Exon inclusion was increased around 1.5 fold in tra2 mutants but

decreased by more than 2 fold in Hfp mutants. These results indicate that the Tra2 protein normally functions to inhibit inclusion of this exon while Hfp acts to favor inclusion. Thus, unlike the M1 intron where Tra2 and Hfp collaborate to repress splicing, the effect of Hfp on exon 12a splicing is opposite to that of Tra2.

Alternative splicing of exon 13a was also tested, but no dramatic effect on its inclusion was seen in RNA from either tra2 or Hfp mutant testes (figure 4-2). It should be noted that these effects of Hfp and Tra2 on splicing in the germline are distinct from those observed earlier in studies on Drosophila S2 cells subject to genotoxic stress (Katzenberger et al., 2009). Thus these factors have different effects on splicing during normal germline development.


A
12
12a
13
w1118
tra2b/tra21
hfp13/Df(hfp)
hfp13/hfp9
12a +
12a -

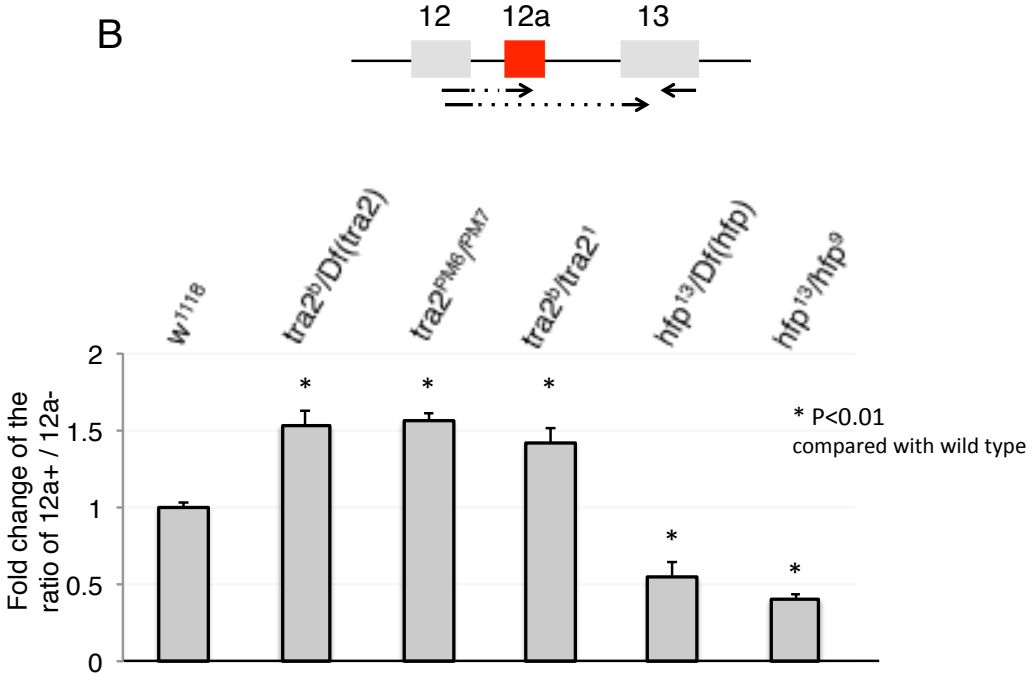

B
12
12a
13
w1118
tra2b/Df(tra2)
tra2PM6/PM7
tra2b/tra21
hfp13/Df(hfp)
hfp13/hfp9
Fold change of the ratio of 12a+ / 12a-
2
1.5
1
0.5
0
*
* P<0.01
compared with wild type

Figure 4-2. Hfp and Tra2 have opposite effect on exon 12a splicing in Drosophila testis

(A) RNA from testis of Drosophila with various genotypes was used for RT-PCR. Primers positions are indicated in the diagram. More exon 12a inclusion was observed in the testis of tra2 mutant flies, while the same exon was reduced in Hfp mutants. (B) Additional genotypes were analyzed by quantitative RT-PCR. The ratio of transcripts with and without exon 12a in the control strain w^{1118} was set at 1.0 and results from other strains and used to normalize results in this comparison. In three different tra2 mutants, the transcripts with exon 12a inclusion were consistently increased, while a 2-fold decrease was observed in Hfp mutants. The Df(2R)Trix chromosome is indicated as Df(tra2). The Df(3L)Ar14-8 chromosome is indicated as Df(hfp).

2. Overexpression of Tra2 in vivo does not cause more exon 12a exclusion

As M1 splicing of Tra2 transcripts in the germline is increased in Hfp mutants (figure 4-3A) these mutants are expected to have higher levels of Tra2 activity in the form of increased Tra2-PC. Therefore the opposite effects of Hfp and Tra2 on Taf1 splicing might be explained in either of two ways illustrated in figure 4-3B. One possibility is that Hfp and Tra2 function in parallel paths to independently regulate exon 12a splicing (figure 4-3B1). Another possibility is that the effect of Hfp on exon 12a is actually a secondary consequence of its effect on M1 repression and Tra2 activity (figure 4-3B2). Without Hfp dependent negative feedback, excess Tra2 is expected and this could lead to the effects opposite to those of loss-of-function Tra2 mutants. To distinguish these two possibilities, exon 12a inclusion was tested after increasing the expression of Tra2-PC in vivo.

We used two approaches to increase Tra2 level in male flies. In the first method we attempted to overexpress Tra2 specifically in primary spermatocytes using Gal4-UAS system. Tra2 expression was driven from a UAS-mycTra2 transgene produced previously in our laboratory and used to study overexpression of Tra2 in the soma (Qi et al., 2006). GAL4 expression was obtained using the Bam-GAL4 transgene known to be active specifically in pre-meiotic spermatogonia and spermatocytes. However, based on RT-PCR experiments, we observed that this system produced only a very small effect on overall Tra2 mRNA levels in the testis (figure 4-

4B). As shown in figure 4-4A, no effects on taf1 exon 12a inclusion were seen in qRT-PCR experiments with Gal4-driven expression of either Tra2 or Hfp.

Because the above results were inconclusive we further explored this splicing regulation working model using several fly strains that contain two copies of a tra2 transgene with the M1 intron has been deleted. As discussed in the Introduction, Tra2 keeps its expression level constant in testis by negatively autoregulating M1 intron splicing (figure 4-5A). This M1 intron deleted transgene is expected to escape negative regulation by Hfp and thus, if Hfp affects Taf1 splicing through its effects on M1, the presence of the transgene should rescue such effects (figure 4-5B,C). Exon 12a inclusion was tested by Q-PCR (figure 4-6). However no difference was observed between the transgenic flies with constitutive Tra2 expression and controls. In this case RT-PCR experiments confirmed that the tra2 mRNA amount were increased within Tra2 overexpression testis. The increased level was comparable to that in Hfp mutant testis (see figure 3-12). This result suggests that the effect of exon 12a splicing we saw in Hfp mutant is not due to the epistasis regulation of tra2 splicing. Hfp functions in parallel with Tra2 on the exon 12a.

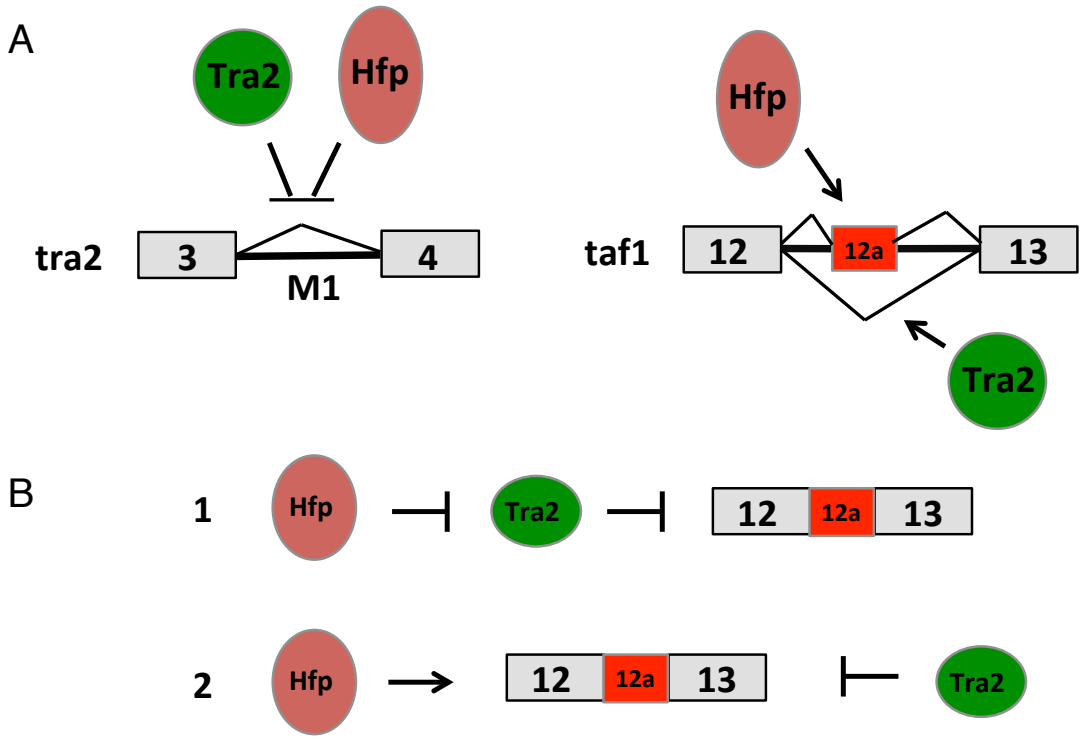


Figure 4-3. Models of RNA splicing regulated by Hfp and Tra2 in the male germline

(A) Hfp and Tra2 are both required to repress M1 intron splicing from tra2 pre-mRNA. Hfp and Tra2 function oppositely in the inclusion of exon 12a splicing in Taf1 mRNA. (B) Two possibilities for the regulation of exon 12a mediated by both Hfp and Tra2. 1) Hfp indirectly affects exon inclusion by negatively regulating Tra2 splicing, and expression of functional Tra2 protein. 2) Hfp and Tra2 function in parallel to control inclusion of the exon.

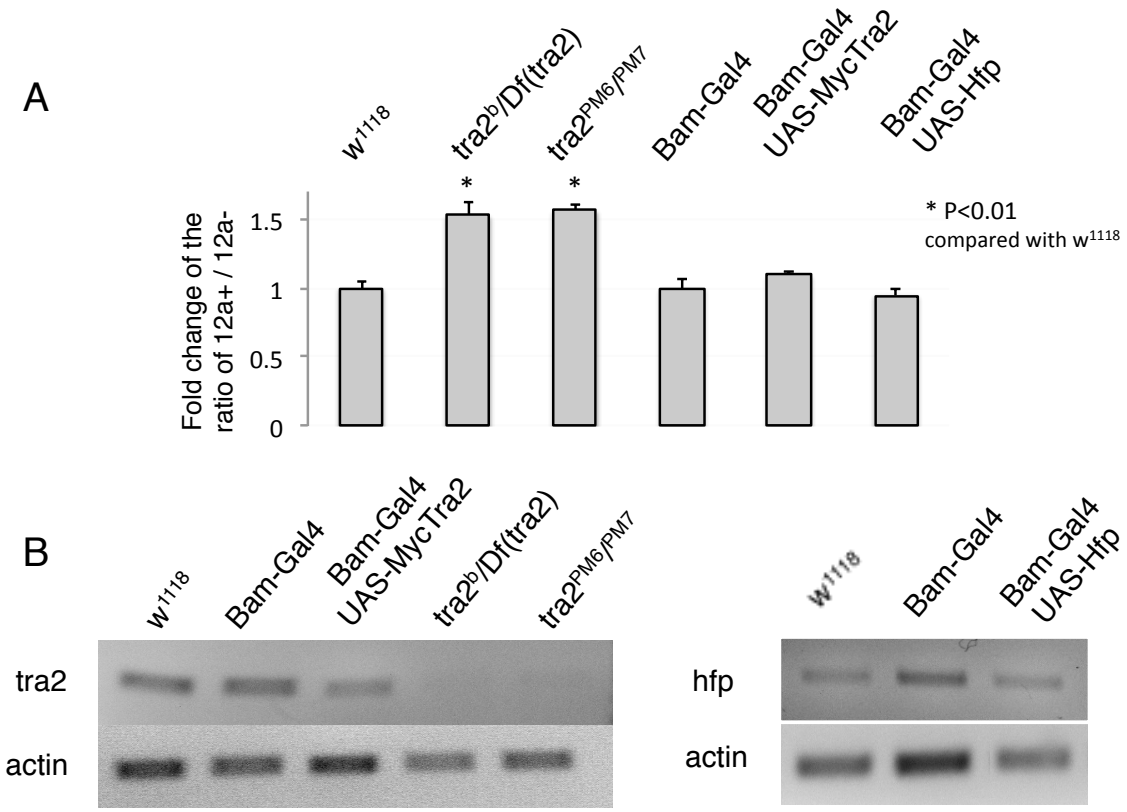
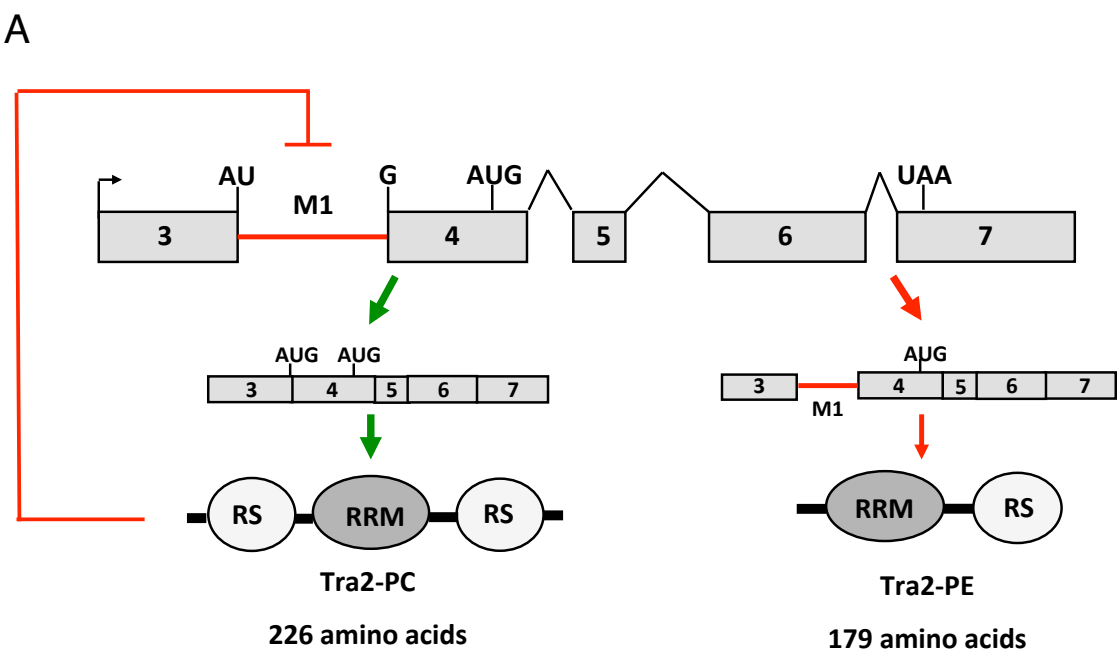


Figure 4-4. The Bam-GAL4 drivert failed to increase expression of mRNAs from UAS-mycTra2 or UAS-Hfp and had no effect on exon 12a splicing (A) Testis RNA from both the wild type control w^{1118} and other control and experimental genotypes was examined quantitative RT-PCR. GAL4-UAS driven expression of Tra2 and Hfp had no effect on exon 12a splicing, compared with Bam-Gal4 control. Error bars represent the standard deviations of the means. (B) Further RT-PCR analysis of RNA from these genotypes indicated that neither Hfp nor Tra2 mRNA levels were significantly increased by the GAL4 and UAS transgenes.


A
AU
M1
G
AUG
UAA
3
4
5
6
7
AUG
AUG
AUG
M1
RS
RRM
RS
RRM
RS
Tra2-PC
226 amino acids
Tra2-PE
179 amino acids

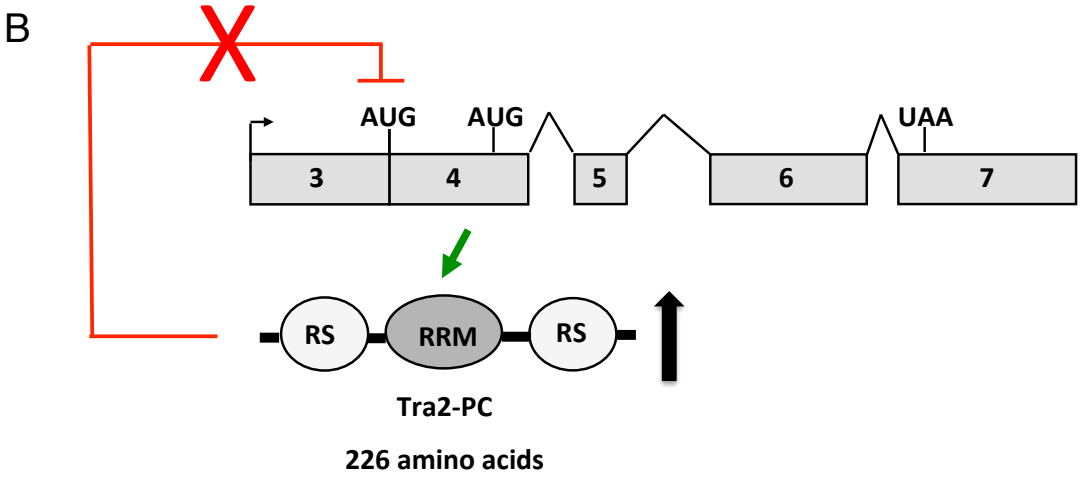

B
AUG
AUG
UAA
3
4
5
6
7
RS
RRM
RS
Tra2-PC
226 amino acids

Figure 4-5. Mutated tra2 gene escapes its negative auto-regulation (A) The diagram showing two mRNA isoforms that are produced by the alternative splicing of tra2 RNA in the male germline. One of the isoforms encodes a functional Tra2 protein with 226 amino acids (also called Tra2-PC). (B) The diagram illustrating how an artificial transgene without the M1 intron can escape negative regulation and keep the Tra2-226 level constantly high in vivo.

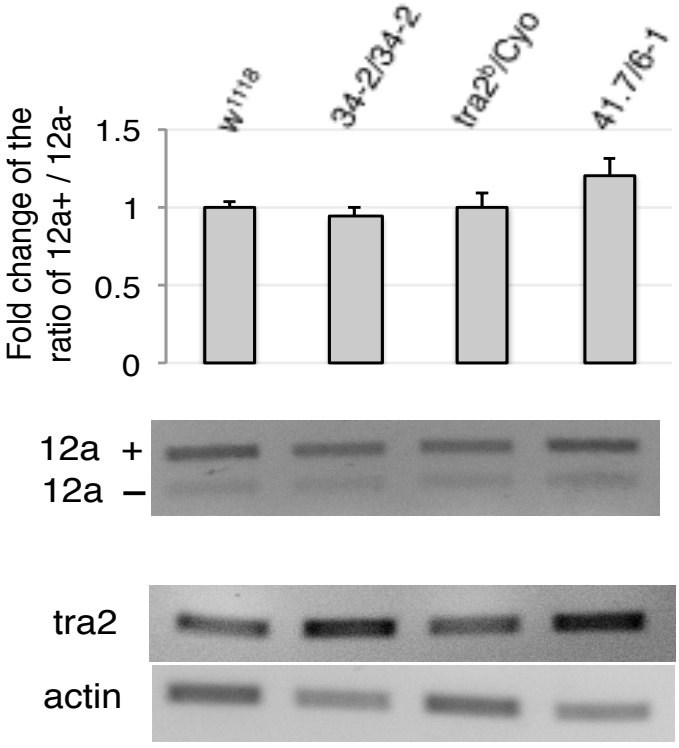


Figure 4-6. Increasing Tra2 in testis does not affect exon 12a splicing

Testis RNA from both w^{1118} and various genotypes was used to do quantitative RT-PCR (graph above) and regular RT-PCR (gel image below) in parallel. Both 34-2/34-2 ($tra2^{+}$ background) and 41.7/6-1 (insertion in the second chromosome that is balanced with *CyO* balancer) are transgenic strains carrying two tra2 transgenes lacking the M1 intron. Although Tra2 mRNA levels were increased, no effect on exon 12a was observed in these two genotypes as compared with wild type (w^{1118}) and $tra2^{b}/Cyo$ controls. Note that the P value of 41.7/6-1 in relation to $tra2^{b}/Cyo$ control is 0.736. Tra2 mRNA levels are shown to be increased in the RT-PCR analysis below the graph. Error bars represent the standard deviations of the means.

3. Hfp and Taf1 are both required for male fertility and the formation of motile sperm

Given its role in Taf1 splicing we next examined whether Hfp is required for normal spermatogenesis. As shown in figure 4-7, two of the Hfp mutant genotypes tested displayed significant male sterility. In addition, among males that displayed fertility there was a dramatic decrease in the number of offspring produced. To determine if sterility was due to an absence of mature sperm we examined the seminal vesicles of sterile Hfp mutants and compared them to wild type. The empty seminal vesicles of the mutants indicate a failure to produce mature motile sperm. We conclude that, like Tra2, Hfp plays an important role in spermatogenesis.

Because Taf1-2 is highly enriched in the male germline and is alternatively spliced under the control of both Hfp and Tra2, we next examined whether Taf1 expression is similarly required for male fertility. To address this issue we first examined available mutations in Taf1. Strong loss-of-function mutations in Taf1 are lethal, and at least one available viable hypomorphic genotype tested produced only fertile males (table 4-1). We therefore turned to RNA interference to produce a tissue specific knockdown of Taf1 function. We took advantage of the Gal4-UAS system to drive Taf1 dsRNA expression specifically in male germ cells. Transgenic flies bearing a construct that produces Taf1 dsRNA from the VAL20 vector were used in these experiments (Haley et al., 2010). This vector is driven by the GAL4 system but is reported to be effective in the male germline and therefore we expected to avoid the low-expression

issues encountered in earlier with germline specific expression experiments. The Gal4 transcriptional activity is driven by Bam promoter which starts transcription in transit amplifying spermatogonia (Chen and McKearin, 2003) which corresponds with the earliest stages where Taf1 is detected (Metcalf and Wassarman, 2007). Using this approach we observed that taf1-RNAi males are completely sterile (figure 4-7).

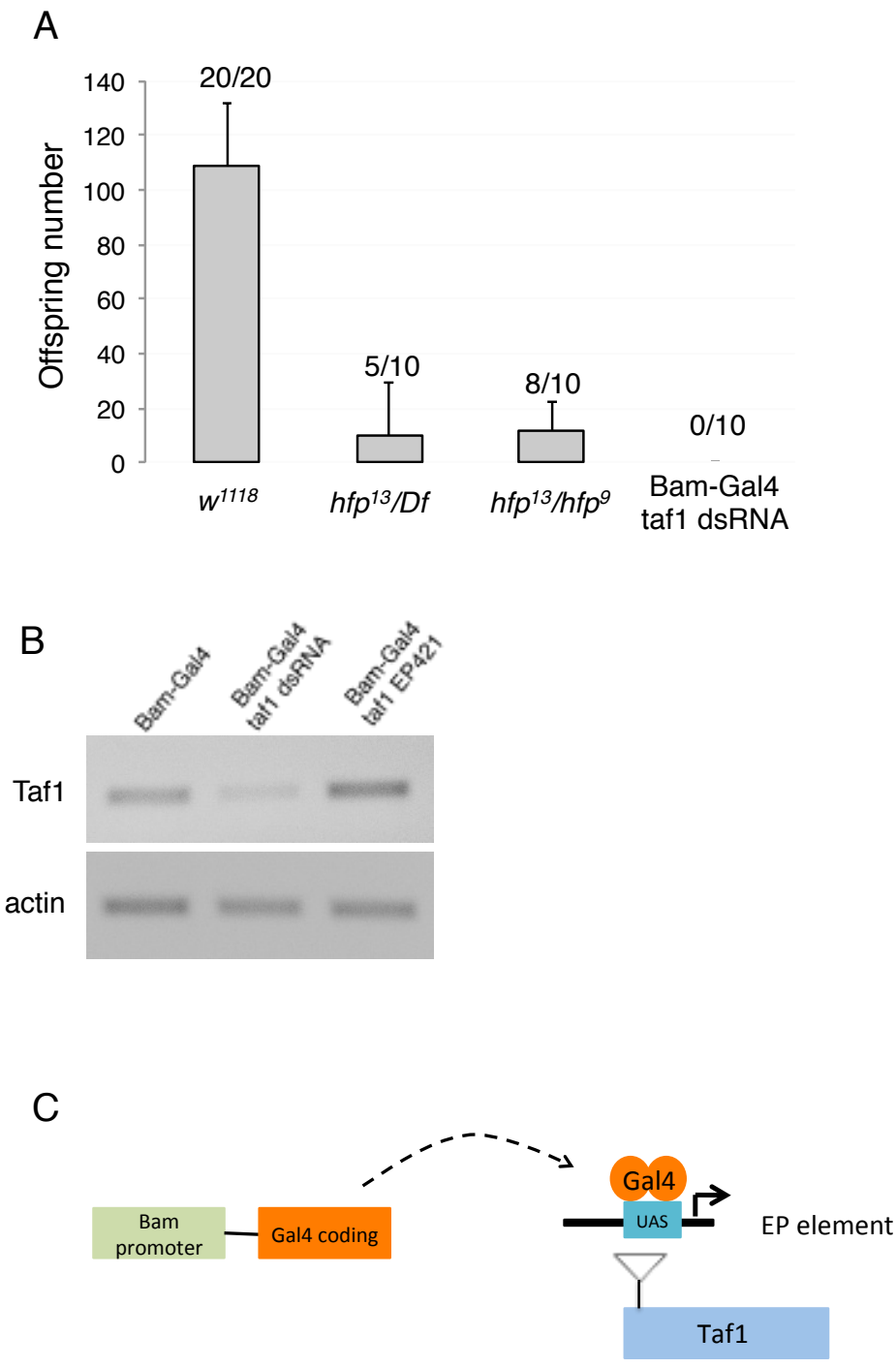

A
Offspring number
140
120
100
80
60
40
20
0
20/20
5/10
8/10
0/10
w1118
hfp13/Df
hfp13/hfp9
Bam-Gal4
taf1 dsRNA
B
Bam-Gal4
Bam-Gal4
taf1 dsRNA
Bam-Gal4
taf1 EP421
Taf1
actin
C
Bam
promoter
Gal4 coding
Gal4
UAS
EP element
Taf1

Figure 4-7. Fertility test of both Hfp and Taf1 mutant males

(A) Offspring from males with various genotypes crossed with wild type virgins were counted and summarized in the graph. The males producing any offspring are defined as fertile. Numbers above the column represent fertile males versus total tested males. Males from both Hfp mutants showed dramatically decreased fertility and complete sterility was observed in taf1 RNAi mutant males. Error bars represent the standard deviations of the means. (B) Expression level in taf1 mRNA in RNAi flies. RT-PCR assays were done with testis RNA from various genotypes. Flies carrying both Bam-Gal4 and the taf1 dsRNA constructs are expected to express taf1 dsRNA in the male germline. Flies carrying Bam-Gal4 and $taf1^{EP421}$ are expected to have increased Taf1 pre-mRNA synthesis as the UAS elements are located immediately upstream of the endogenous Taf1 gene, as illustrated in (C).

Males	No. fertile/No. tested	No. of progeny per fertile male
w^{1118}	10/10	79.4 ± 15.1
$taf1^{R14}/taf1^{EP421}$	9/10	81 ± 10.45
$taf1^{1}/taf1^{EP421}$	lethal	

Table 4-1. Fertility tests of Taf1 mutants

No effect on the fertility of $taf1^{R14}/taf1^{EP421}$ mutant was observed. The other mutant $taf1^{1}/taf1^{EP421}$ is lethal.

4. Half pint is required for a normal number of transit amplifying mitotic divisions in spermatogonia

Spermatogenesis in Drosophila starts with production of gonial cells from germline stem cells located at the anterior tip of the testis. These gonial cells will divide four times in synchrony with incomplete cytokinesis to form a cyst of 16 interconnected cells. The cyst is surrounded by two somatic cyst cells. These divisions are referred to as transit amplifying mitotic divisions because they amplify the number of products from the stem cell. Once the spermatogonia number reaches 16, they will stop dividing and start to grow. By the time spermatocytes enter meiosis they have increased in volume by 25 times (Insco et al., 2009). The whole process is illustrated in figure 4-8.

Examination of early spermatogenesis revealed that in each of several half pint mutant genotypes (*hfp^{13}/hfp^{9}* see figure 4-9, *hfp^{13}/hfp^{3058}*, *$hfp^{13}/Df(3L)Ar14-8$* data not shown) the majority of spermatocyte cysts produced 8 rather than the usual 16 germ cells. This suggests that, like in the female germline, Hfp is required to promote a fourth mitotic cell division. Examination of the morphology in these cysts indicates that the cells within them have features distinctive of spermatocytes (large and round nuclei and prominent nucleoli, no sign of dividing). Moreover in a manner similar to 16 cell cysts, they grow dramatically after mitosis is completed and are able to enter meiosis upon completion of the growth phase (see below). These results indicate that Hfp is required to determine the number of spermatocytes/cyst but are not

required for other visible pre-meiotic events. We also examined spermatocytes from tra2 mutants, but consistent with previous reports, the loss of function for this gene had no visible effect the number of mitotic divisions or on spermatogenesis prior to meiosis (figure 4-10).

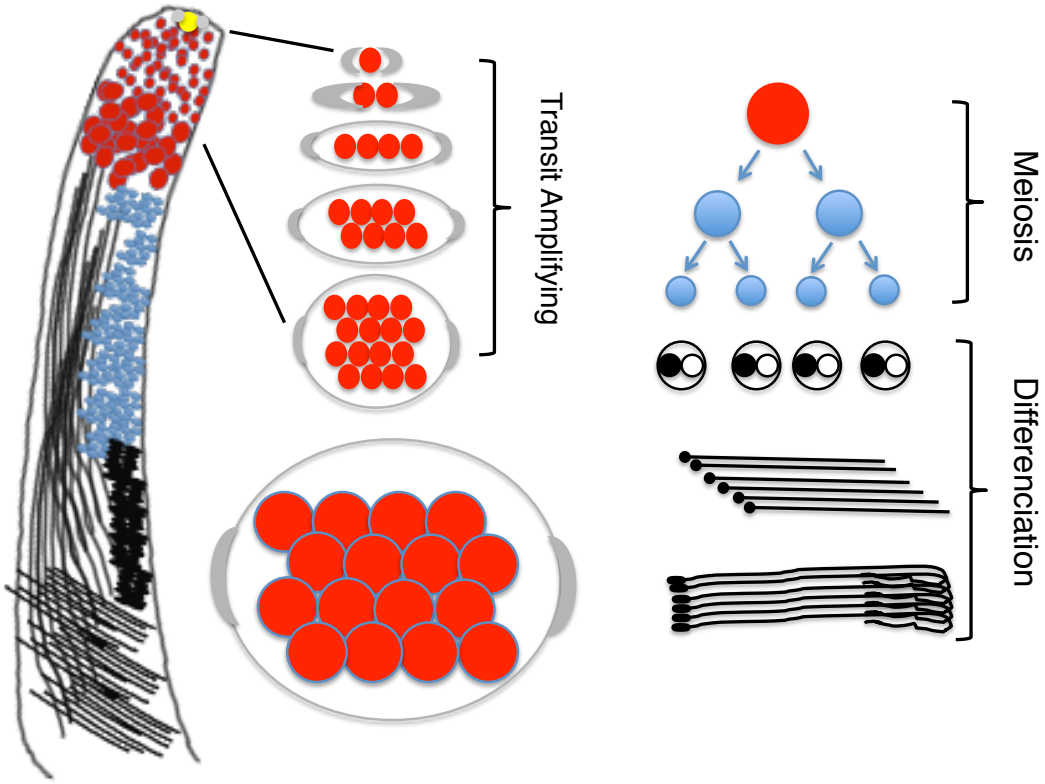


Figure 4-8. Illustration of spermatogenesis in Drosophila

Germ cells within the adult testis are illustrated on the left. The yellow circle at the tip represents the germline stem cell. Gray circles around it represent cyst cell progenitor. Red circles represent spermatocytes in the stages of both mitosis and post-mitotic growth. Blue circles correspond to spermatocytes in meiosis. Black circles and lines represent spermatids in post-meiotic differentiation stages.

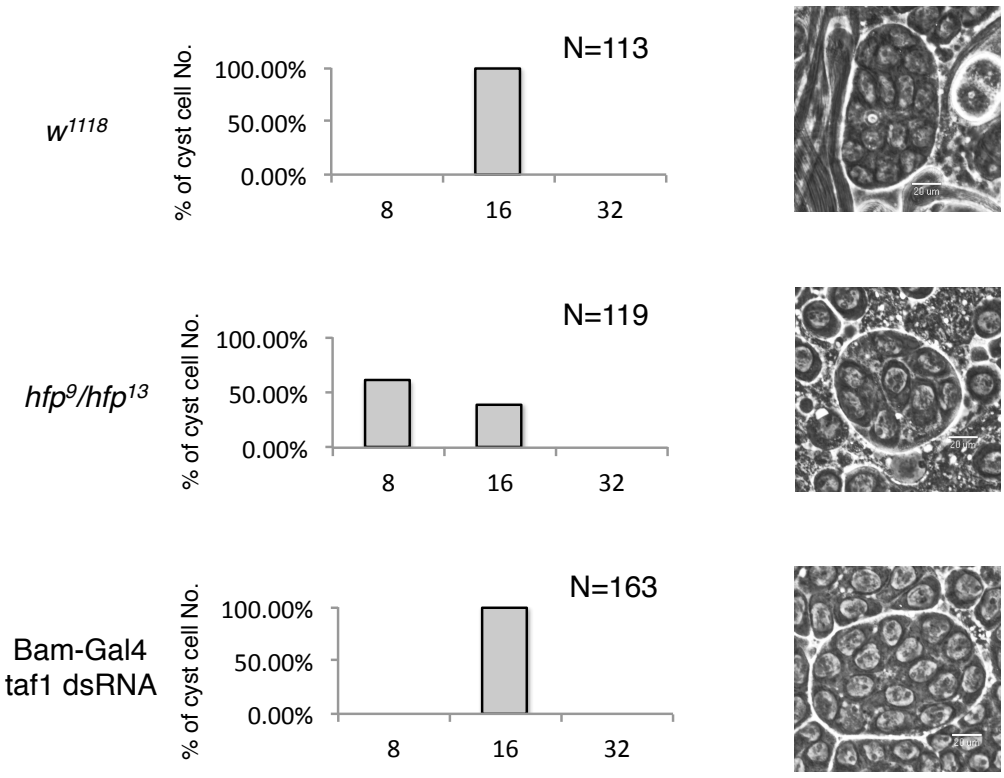


Figure 4-9. Eight-cell spermatocyte cysts were produced in the testis of Hfp mutants

Testes from Drosophila adults of various genotypes were dissected and gently opened under pressure of a coverslip. Spermatocyte cysts were counted under phase contrast microscope. The percentage of cysts with various number of spermatocytes in each mutant testis are shown in graphs. In *hfp*^{13}*/hfp*^{9} mutant testis more than half of the cysts contained 8 spermatocytes, while all the cysts in taf1 RNAi testis contained 16 spermatocytes. The number of cysts counted (N) is indicated in each graph. Typical cyst phase contrast images are shown on the right side of the graphs.

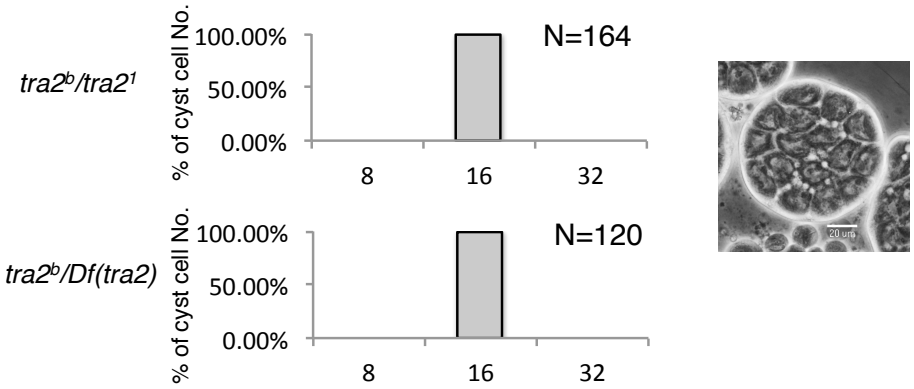


Figure 4-10. The testis of Tra2 mutant flies contain cysts with normal numbers of spermatocyte

Testes from two tra2 mutant genotypes were dissected and peeled open. Spermatocyte cysts were counted under phase contrast microscope. Percent of cysts with different number of spermatocytes in each mutant testis are shown in graphs. The number of spermatocytes in each cyst is normally 16, and no cyst with abnormal numbers of spermatocytes was observed. Total cysts counted for each genotype (N) are indicated. Phase contrast images of typical cysts are on the right side of the graphs. The Df(2R)Trix chromosome is indicated as Df(tra2).

5. Half Pint is required for normal spermatogenesis

To further investigate how spermatogenesis is altered in Hfp mutants we examined the morphology of mature spermatids using, propidium iodide to stain DNA and antibodies against beta-tubulin, which is a major structural component of the spermatid tails. In wild type testes, as shown in figure 4-11, each cyst matures to form a bundle of aligned elongated spermatids with condensed nuclei. These accumulate primarily near the posterior end of testis (indicated with the arrow) where individualized mature sperm will eventually swim into the seminal vesicle. Prior to the completion of maturation, spermatid are nicely aligned with highly condensed needle like nuclei, identified with PI staining, and long tails identified by beta-tubulin staining (figure 4-11). However in Hfp mutants, clusters of aligned nuclei were not observed. Instead, the number of condensed nuclei is significantly diminished (figure 4-11). Of the scattered nuclei observed, very few were elongated to form the needle-like shape formed in wild type. In addition the regular spatial relationship between nuclei and the tails found within normal cysts was lost. Based on beta-tubulin staining, tails appeared to be irregularly formed and nuclei were distributed within tails randomly (figure 4-11).

To visualize individual cysts we tried squashing testes stained by anti beta-tubulin antibody and propidium iodide. As shown in figure 4-12, wild type testis showed nice alignment between spermatids that included both the nuclei and tails. But condensed nuclear bundles were not observed in Hfp mutant. Instead, large and irregularly shaped nuclei were scattered and no clear

connection was observed with tails. These observations suggest that during spermatid differentiation, the coordination development of spermatid heads and tails is impaired.

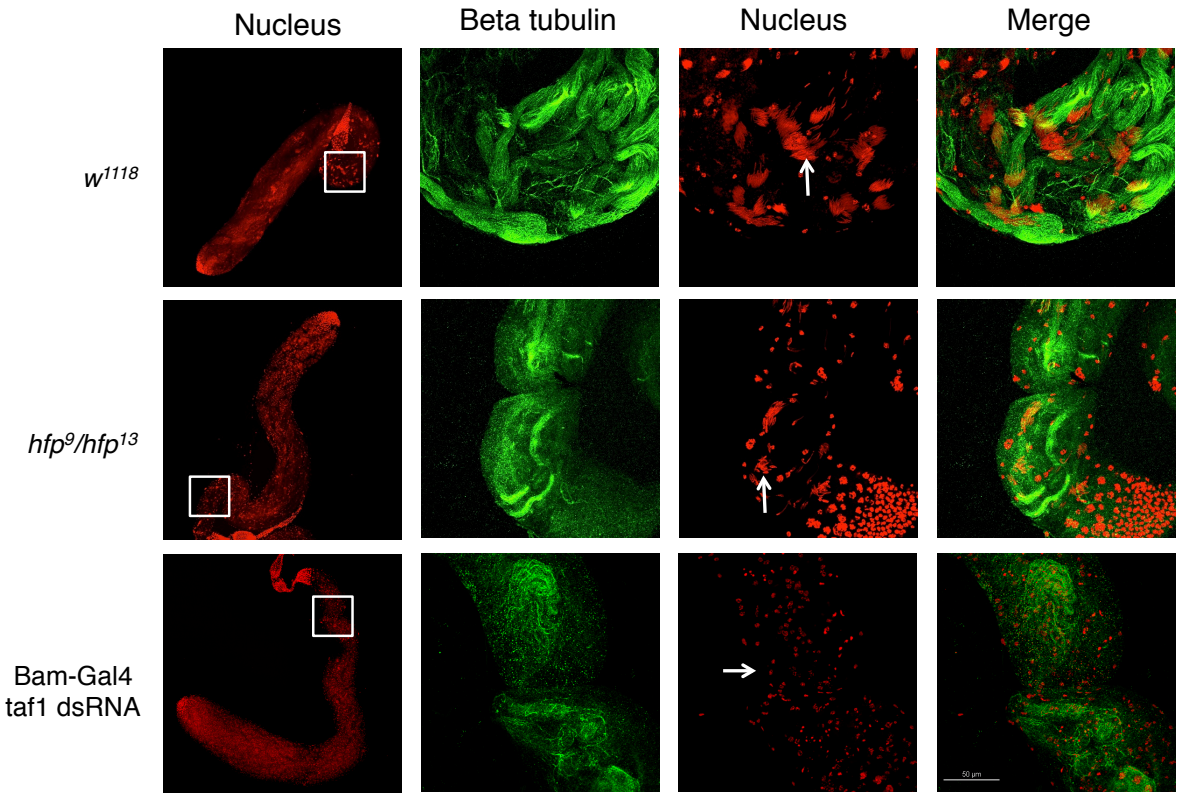

Nucleus
Beta tubulin
Nucleus
Merge
w1118
hfp9/hfp13
Bam-Gal4
taf1 dsRNA

Figure 4-11. The differentiation of spermatid is impaired in both Hfp and Taf1 mutant testis

Whole testis were dissected and stained with anti-beta tubulin antibody (green) and PI (red). Less sperm head bundles and tails were observed in both Hfp and Taf1 mutants. The magnified field from each image is indicated by the white square frame on the left. Few or no clusters of spermatid nuclei and fewer tails were seen in both Hfp and Taf1 mutants. Further, sperm nuclei were not found at the end of the cluster but rather were distributed throughout the cyst. The sperm nuclei showed irregular shaped instead of needle shaped in wild type.

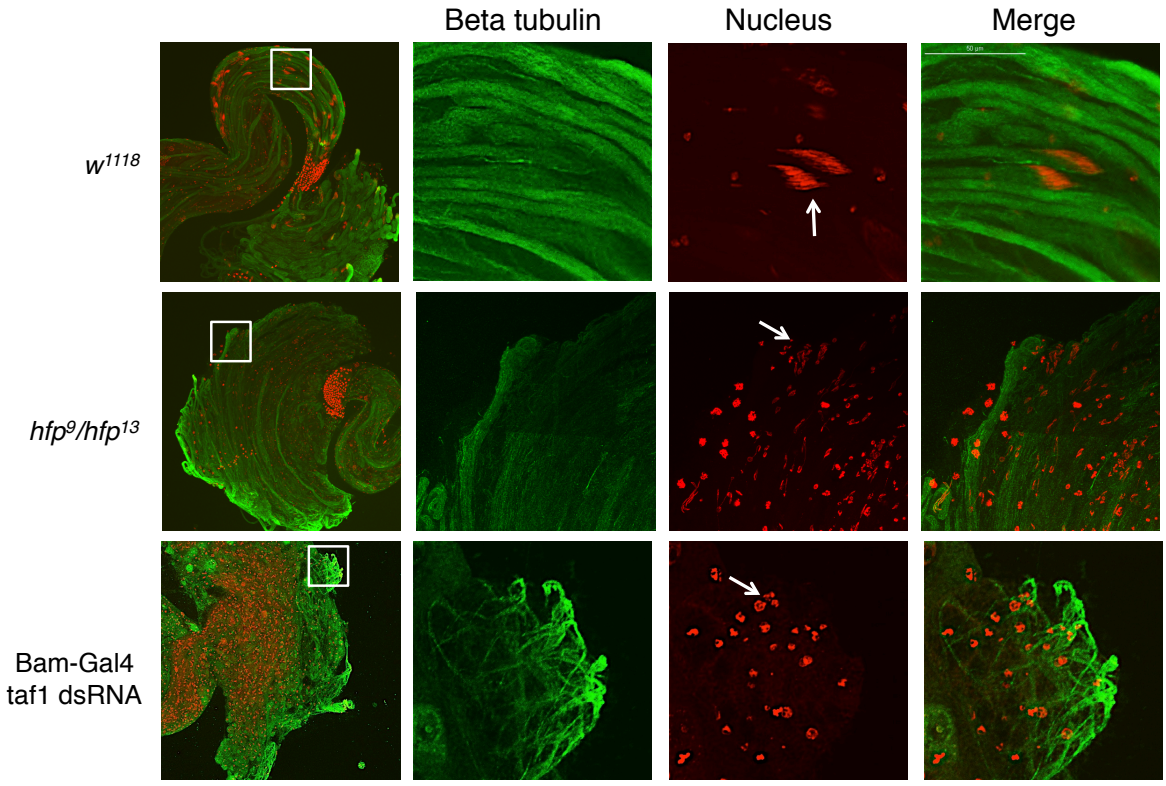

Beta tubulin
Nucleus
Merge
w1118
hfp9/hfp13
Bam-Gal4
taf1 dsRNA

Figure 4-12. Hfp and Taf1 mutant males contain less sperm bundles and tails

Immunostaining like in the previous figure is shown on spermatid cysts from squashed testes. Fewer recognizable nuclear bundles and tails were observed in both Hfp and Taf1 mutants. The magnified field from each image was indicated with white square frame on the left. Similar phenotypes with that in figure 4-11 were observed.

6. Hfp is required for correct meiosis during spermatogenesis

The failure to produce normal mature spermatids could be due to aberrations in spermatid differentiation or to defects that occur in meiosis. To examine how these processes are affected by Hfp, we characterized cellular phenotypes of cysts at earlier stages.

Examination of the testis as a whole showed that, in relation to wild type, Hfp mutant testis was in a relatively smaller size, which could be due to the observation that more than half of the cysts contained 8 spermatocytes instead of 16. Also Hfp mutants contained less sperm tails as indicated in wild type, which is consistent with the results of immunostaining assay, and suggests a relatively low number of mature spermatids (figure 4-13).

After meiosis is completed, 64 spermatogonia are normally found in each cyst. The mitochondria in the cytoplasm of each cell aggregate and fuse with each other to form a structure called a Nebenkern, which is a dark spherical structure under phase contrast. In normal testes, a single Nebenkern is found closely associated with the cell nucleus and is about the same size, as shown in the w^{1118} cyst in figure 4-14. Changes in this arrangement are observed in Hfp mutants and are indicative of mistakes in meiosis. For example, we observed that Hfp mutant spermatids associated with multiple Nebenkern suggesting a failure in cytokinesis (figure 4-14). In addition we found that nuclei of varying sizes were often found in postmeiotic cysts of Hfp mutants and that their size often differed substantially from that of the Nebenkern and from that of nuclei found in normal cysts at a similar stage. This variance in

size is an indication of chromosomal aneuploidy and suggests that the mutant cells fail to undergo normal chromosome segregation. Thus it is likely that Hfp mutants suffer from multiple defects in the process of meiosis in the male germline.

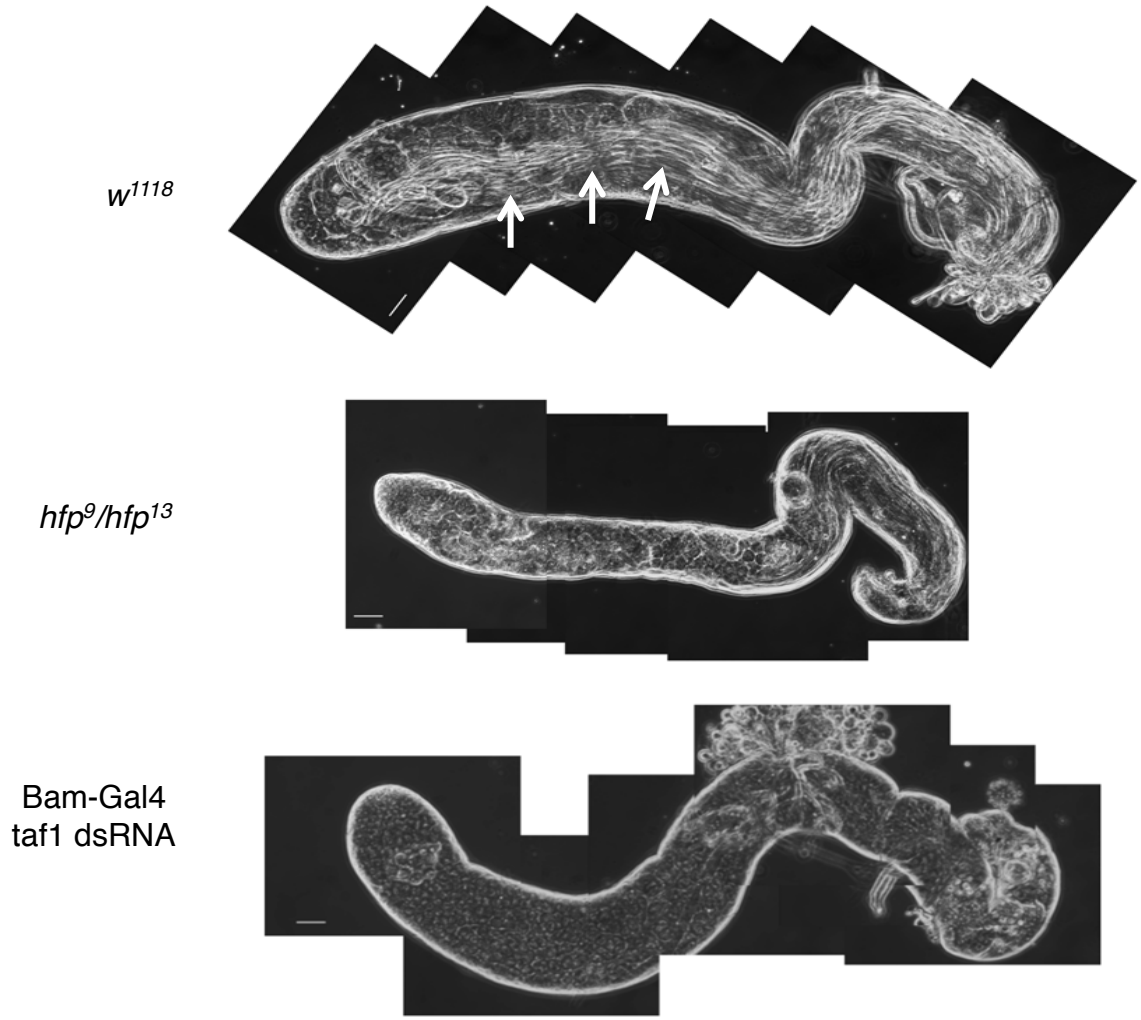
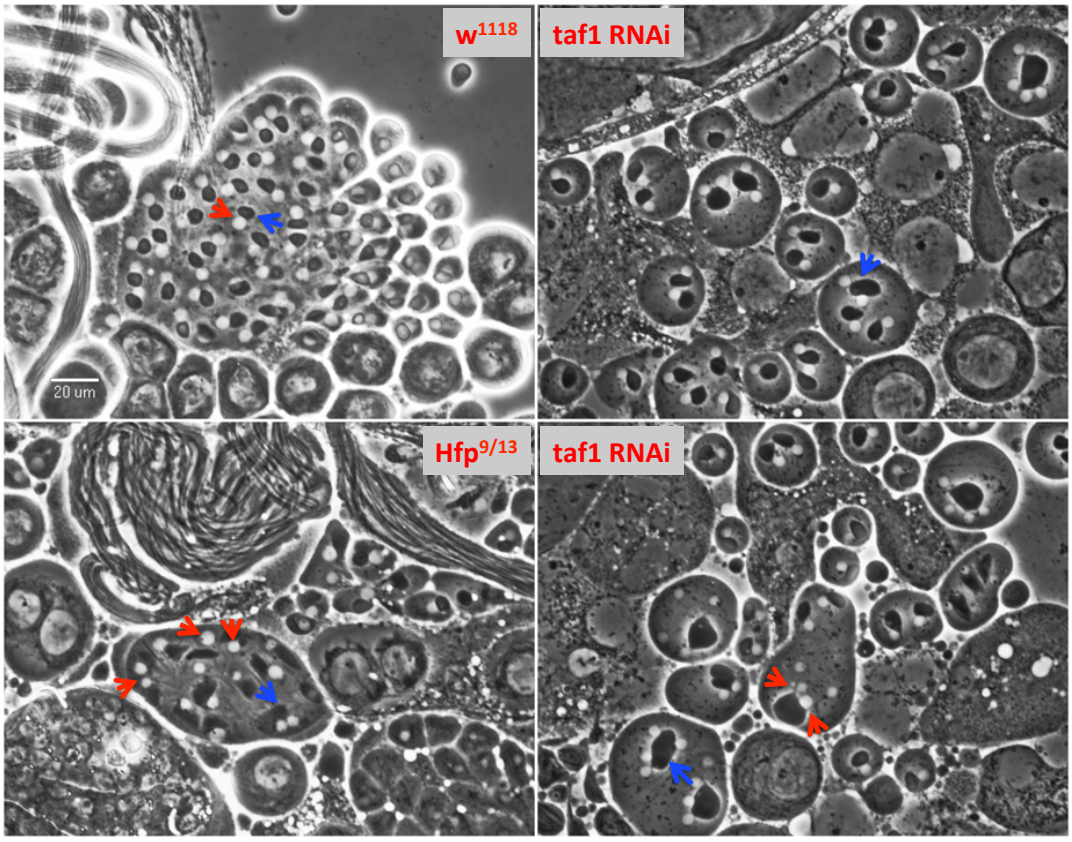


Figure 4-13. Decreased amount of sperm tails developed within Hfp and Taf1 mutant testis

Testes from one-day old male were dissected and images were taken with phase contrast microscope. Whole testis images were rebuilt with the same magnification scale. Genotypes are labeled on the left of the images. More white fibers are seen in wild type testis as indicated by the arrows.

A



B

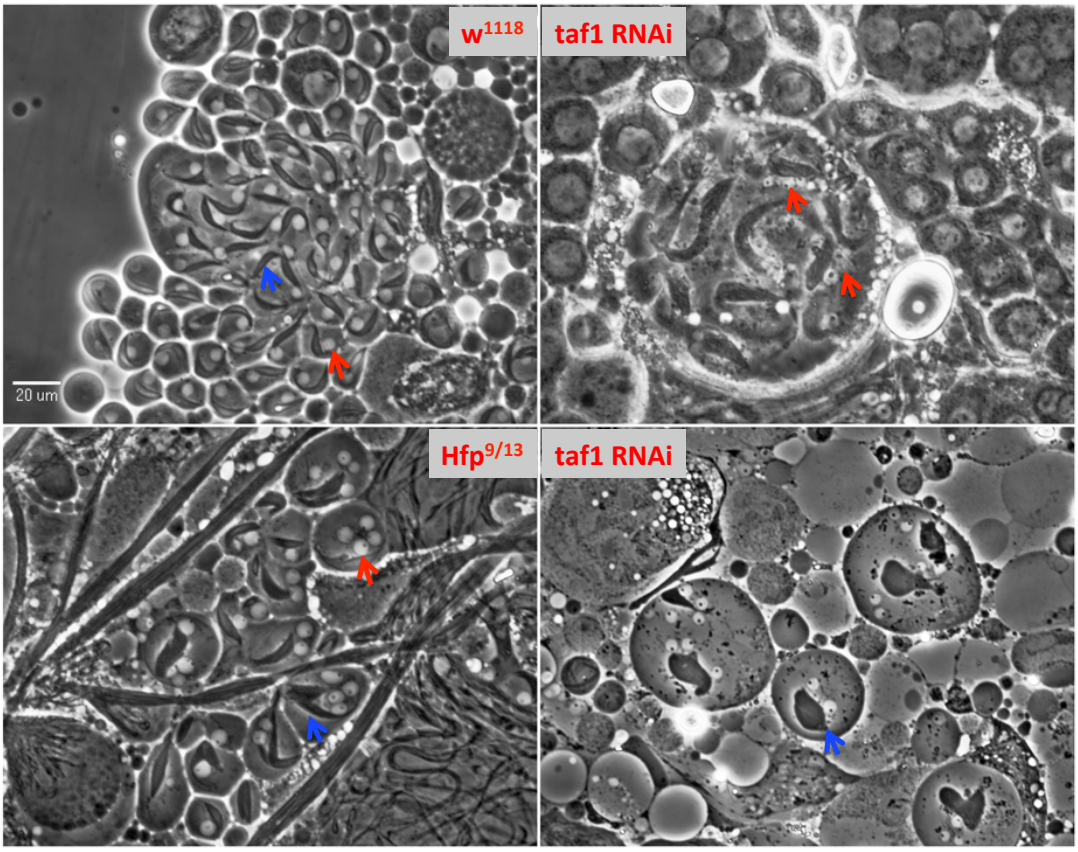


Figure 4-14. Evidence of meiotic defects in Hfp and Taf1 mutant testes

(A) Abnormal cytological phenotypes were observed in the onion stages of spermatid differentiation. Testes were dissected and squashed in the dissection buffer. Images were taken with phase contrast microscope. Nucleus are indicated with red arrows, and Nebenkern are indicated with blue arrows. Variably sized nuclei (white circles) were seen in both Hfp and Taf1 mutants, as indicated with red arrows. Multiple nucleus associated with one cytoplasm were also seen in both Hfp and Taf1 mutants as indicated with blue arrows. (B) In slightly later stages of spermatid differentiation Nebenkerns begin elongation as shown in the wild type control (w^{1118}), but this abnormal in cysts from either Hfp or Taf1 mutants. In these cells Nebenkerns had irregular shapes and were associated with multiple small nuclei.

7. Comparative analysis of spermatogenesis phenotypes in Hfp, Tra2 and Taf1 mutants

Analysis of splicing in Hfp and Tra2 mutants indicates that these factors have opposite effects on the regulation of taf1 exon 12a splicing. Specifically, Hfp was observed to be required for normal levels of the exons inclusion which in turn leads to the expression of the testis-specific Taf1-2 protein isoform. If Taf1-2 plays an important role in germ line development, we might expect that Taf1 loss-of-function genotypes would display phenotypes that overlap those of Hfp mutants. Interestingly it is reported that 8 cell egg chambers, like those observed in Hfp females have been observed in the oogenesis of Taf1 mutants (Wassarman et al., 2000). But how the male germ cell development is affected by loss of Taf1 has not been reported.

To address this issue taf1-RNAi whole testis were examined by phase contrast, as seen in figure 4-13, Taf1 loss-of-function gonads was primarily filled with spermytocytes and relatively few mature spermatids are seen. This is consistent with the result from whole testis staining with anti beta-tubulin antibody. As shown in figure 4-11 and 4-12, few sperm tails were formed and almost no spermatid head bundles were seen in the whole testis of taf1-RNAi mutant. The phenotype is similar when squashed testis was stained. Spermatid tails are short, poor aligned with each other. Nuclei are relatively large and irregularly shaped in contrast to the needle-shaped nuclei seen in wild type. Like in Hfp mutant spermatids were not well aligned and nuclei were observed to be distributed throughout the tails.

Under phase contrast, cytogenetic defects can be seen in the stages after meiosis (figure 4-14). Relatively large nuclei were seen in the stage of condensation. At the same time, uneven divisions produced many daughter nuclei with bigger and smaller sizes clustered within the same cyst. Some daughter nucleus shared the same Nebenkern, while some cells lost nucleus but has only Nebenkern left. However no 8-spermatocyte cysts was seen in taf1-RNAi testes (figure 4-9).

These observations together suggest that Hfp and Taf1 mutants share some common phenotypes during spermatogenesis. And the phenotypes in taf1-RNAi mutant are more severe.

Unlike Hfp and Taf1, in both of two well studied Tra2 genotypes tested, *tra2^{B}/tra2^{1}* and *tra2^{B}/Df(2R)Trix*, only 16-spermatocyte cyst was seen as shown in figure 4-10. Interestingly, this mutant also affects sperm nuclear morphology as described previously (Belote and Baker, 1983), but no effects on meiosis were observed in these testes.

8. Meiosis-related markers are affected similarly in Hfp and Taf1 mutants

Since similar meiosis defects were observed in both Hfp and Taf1 mutant testis, we tested whether the molecular markers involved in meiosis were similarly affected by these two factors. The markers tested included the meiosis related genes *always early (aly), cannonball (can), twine, cyclin A and cyclin B* as well as the spermiogenesis-related genes *don juan (dj) and fuzzy onions*

(fzo). These particular markers were selected based on their previous characterization in studies on another meiosis-related gene mutant in the germline (Ayyar, 2003). Gene expression levels were examined by qRT-PCR in RNAs from taf1 RNAi and Hfp mutant testis. Mutations in the *always early* (*aly)* and *cannonball* (*can*) genes both produce meiosis-arrested phenotypes and these factors are thought to regulate transcription of target genes involved in entering meiosis. Expressions of *cyclin A*, *cyclin B* and *twine* are indicative of various steps related to meiosis initiation. *Don Juan* (*dj*) and *fuzzy onions* (*fzo*) are both genes required for the differentiation of functional sperm. As shown in figure 4-15, *cyclin A* and *cyclin B* were both significantly increased in Taf1 mutant, similar but rather milder effects were seen in Hfp mutant. Twine has no statistically significant increase in both Hfp and Taf1 mutants, but its trend of changes are similar in the two mutants. Since *cyclin A* and *twine* RNA are both accumulated in late G2/M spermytocytes, the increase observed in their level is consistent with microscopic observations that these mutant testes have an increased proportion of pre-meiotic spermytocytes (figure 4-13). Transcripts from *dj* were dramatically decreased in both Taf1 and Hfp mutant. Since it was reported that *dj* is involved in the spermatid differentiation and maturation (Santel et al., 1998), its decreased level in both mutants is consistent with the staining result that sperm in both mutants showed developmental defects. However *can* was only down regulated in Hfp mutant. *Cannonball* is another testis-specific taf gene that is known to regulate downstream targets involved in meiosis (White-Cooper et al., 1998).

Its decreased level only observed in Hfp mutant suggests that Hfp might be in a higher position of the hierarchy of gene expression during spermatogenesis, but Taf1 is a downstream target. Other markers tested were not significantly affected in either mutant. This observation differs from that reported for mutations affecting genes encoding other germline specific Taf proteins which have more global effects on factors involved in meiosis and spermiogenesis (Ayyar, 2003; Hiller et al., 2004).

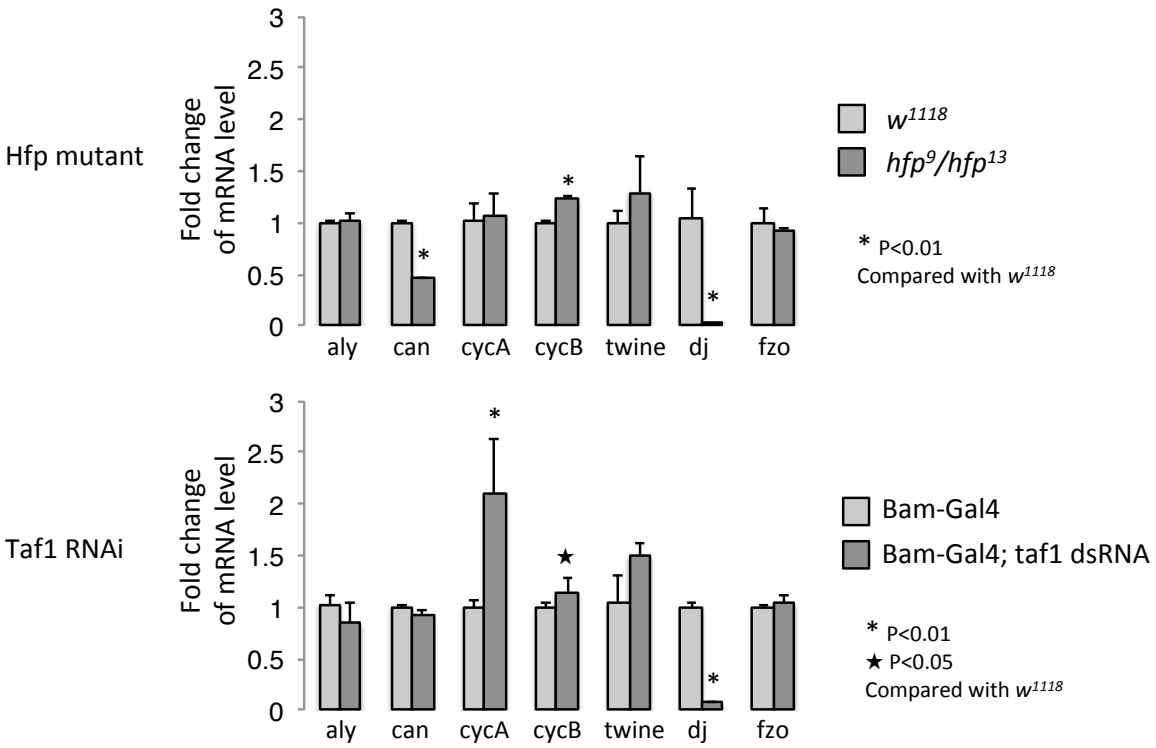


Figure 4-15. Expression levels of genes that are involved in meiosis in both Hfp and Taf1 mutant testis

Gene expression levels of various marker genes are indicated as determined by qRT-PCR performed on RNA from dissected testes. All samples were normalized to parallel amplifications of the ribosomal protein L32 mRNA. Error bars represent the standard deviations of the means.

9. Examination of the fertility of Hfp mutant males affected by forced expression of Taf1

If the phenotypic effects of Hfp on meiosis and spermiogenesis are due to it's effects on the expression of Taf1-2, then the forced expression of this product is expected to suppress (rescue) the sterility phenotypes of Hfp mutants. The *taf1*^{EP421} allele contains an EP element inserted in the first exon of Taf1. The EP element is a P element derived construct that contains UAS target sequences of Gal4 transcriptional factor at its 3' end. In the presence of GAL4, the gene downstream of the EP element will be transcribed in a tissue specific manner. To test whether a forced increase in Taf1 in spermatocytes results in rescue of the Hfp mutant phenotype we combined this EP element with the Bam-GAL4 driver transgene in a Hfp mutant background. To build such a strain, a genetic recombination strategy was used to combine four elements within one genome: two Hfp mutant alleles, the Bam-Gal4 transgene and taf1^{EP421}. The genetic scheme we used is shown in figure 4-16 and the increased taf1 expression level in the males produced was confirmed in results that are shown in figure 4-7.

The fertility of four element male flies were tested in crosses with wild type virgins. After mating, the genotypes of all male parents were verified by PCR (figure 4-17). Fertility test results are summarized in figure 4-18. The mean numbers of progeny in these crosses were 21.2 and 16.7 compared with *Bam-Gal4; hfp*^{9}*/hfp*^{13}. This suggests that increasing overall Taf1 transcription in this way had little, if any, effect on the fertility of Hfp

males. However we did note differences in the frequencies of males producing larger numbers of progeny. All wild type males tested produced more than 60 offspring, but individuals with this level of fecundity were not observed among Hfp mutants that carried Bam-Gal4. In contrast 8-15% of Hfp mutant males with Taf1 overexpression produced more than 60 offspring. This suggests that although the fertility of most mutant males is unaffected by increased Taf1 expression, a small fraction of individuals has significantly increased fecundity. These observations warrant further investigation in the future as they may indicate an important role for the regulatory relationship between Hfp and Taf1 splicing.

Part A

Male: *taf1^{EP421}/TM3, Sb* X Female: *hfp^{13}/TM6B, Hu*

↙

Virgin: *hfp^{13}/taf1^{EP421}* (Sb^{+} and Hu^{+}) X Male: *Df/TM6C,Sb*

↙

20 individuals Male: *hfp^{13}, taf1^{EP421}/TM6C, Sb* X Virgin: *Ly/TM6B, Hu*

↙

PCR Hu males (without Ly) from each line
For hfp^{13} and taf1^{EP421} – identify double positive lines
hfp^{13}, taf1^{EP421}/TM6B, Hu

↓

virgins from the same double positive line X other males from double positive lines

↓

Stock: *hfp^{13}, taf1^{EP421}/TM6C, Sb*

Part B

Male: *hfp^{9}/TM6B, Hu* X Female : *w, P[m-w+, bam-Gal4-Vp16]; +/+*

↙

Virgin: *Bam-Gal4/+; hfp^{9}/+* (Hu^{+}) X Male: *+/Y; Df/TM6C,Sb*

↙

20 individuals Male: *Bam-Gal4/Y; hfp^{9}/TM6C, Sb* X Virgin: *Ly/TM6B, Hu*

↙

PCR Hu males (without Ly) from each line
for Bam-Gal4 and hfp^{9} – identify double positive lines
Bam-Gal4/Y; hfp^{9}/TM6B, Hu

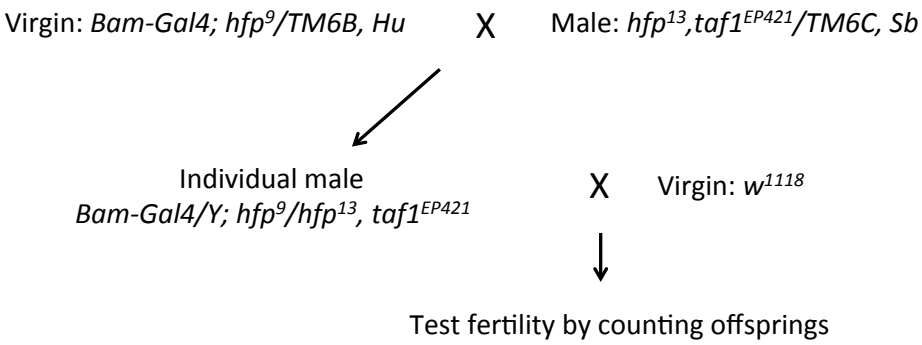
↓

virgins from the same double positive line X other males from double positive lines

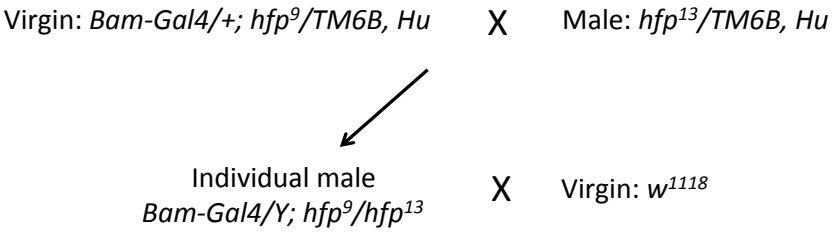
↓

Stock: *Bam-Gal4; hfp^{9}/TM6B, Hu*

Part C



Part D



Part E

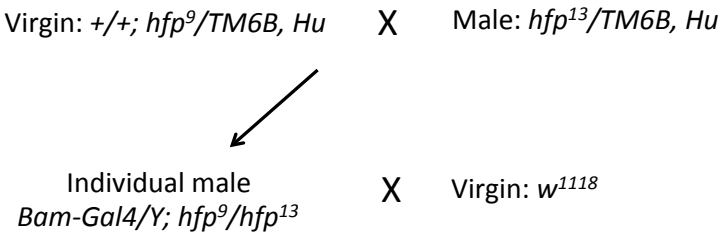


Figure 4-16. The schemes for fly crosses to generate strains with Taf1 overexpression in a Hfp mutant background

The genetic scheme for producing the fly strain with both hfp^{13} and $taf1^{EP421}$ is shown in part A. The production of the other fly strains with both hfp^{9} and Bam-Gal4 is shown in part B. The processes of producing other controls are shown in part C-E.

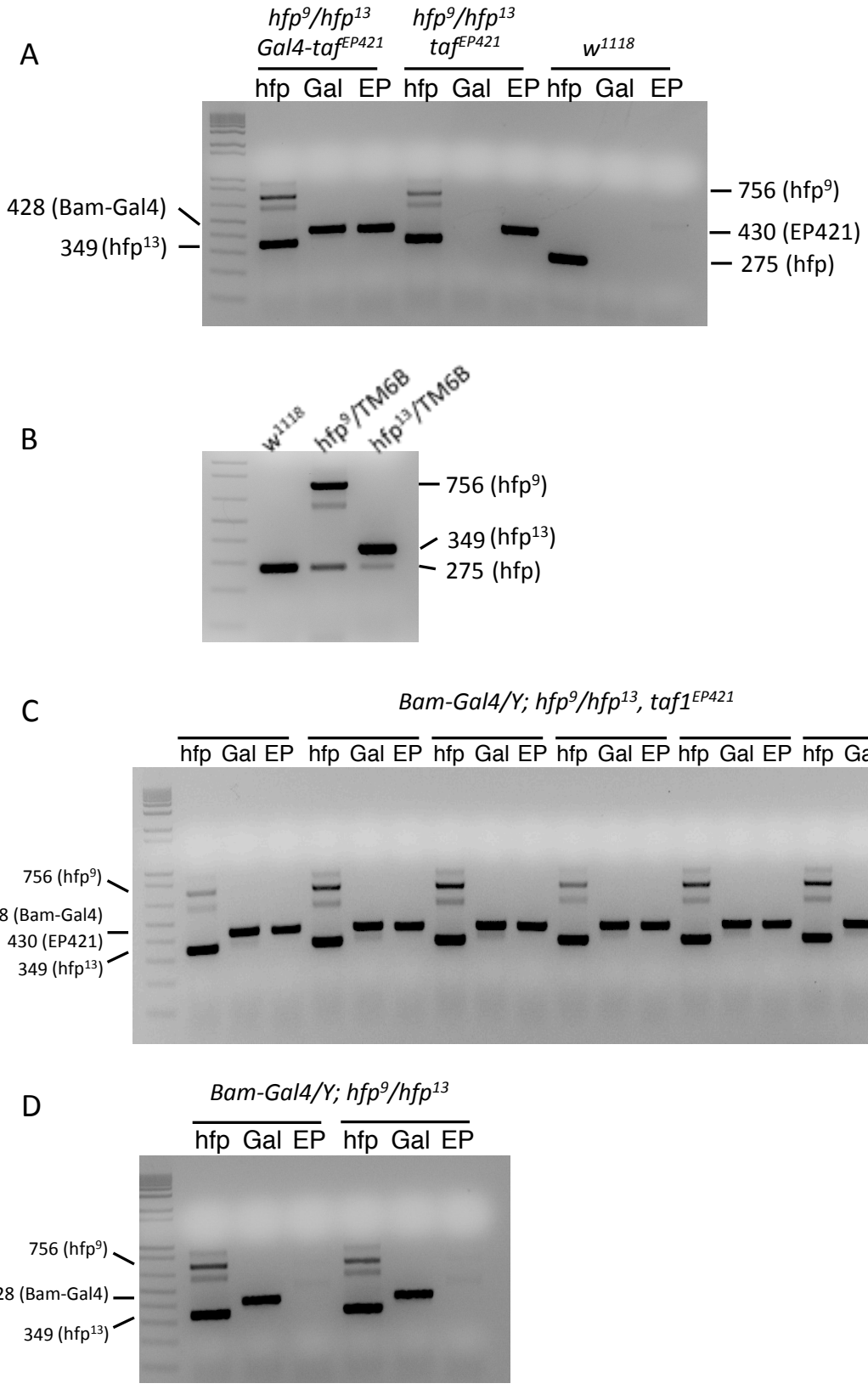
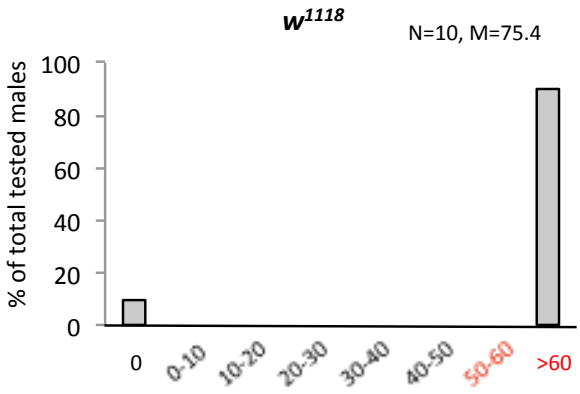
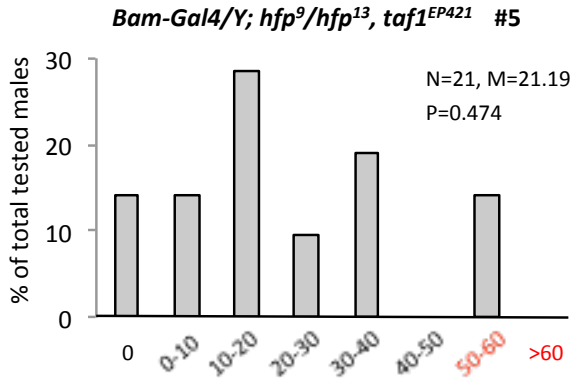
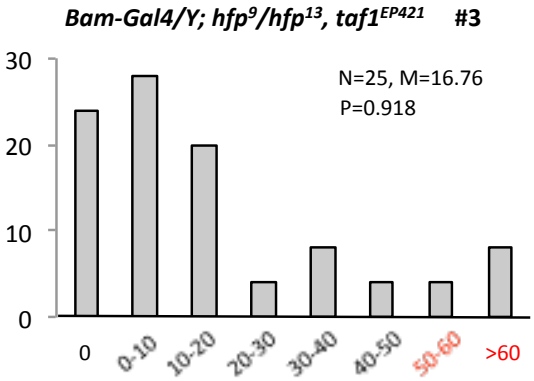

A
hfp9/hfp13 Gal4-taf EP421
hfp9/hfp13 taf EP421
w1118
hfp Gal EP hfp Gal EP hfp Gal EP
428 (Bam-Gal4)
349 (hfp13)
756 (hfp9)
430 (EP421)
275 (hfp)
B
w1118
hfp9/TM6B
hfp13/TM6B
756 (hfp9)
349 (hfp13)
275 (hfp)
C
Bam-Gal4/Y; hfp9/hfp13, taf1 EP421
hfp Gal EP hfp Gal EP hfp Gal EP hfp Gal EP hfp Gal EP hfp Gal EP
756 (hfp9)
428 (Bam-Gal4)
430 (EP421)
349 (hfp13)
D
Bam-Gal4/Y; hfp9/hfp13
hfp Gal EP hfp Gal EP
756 (hfp9)
428 (Bam-Gal4)
349 (hfp13)

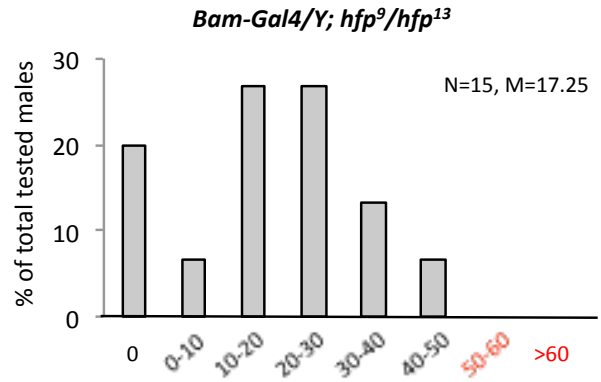
Figure 4-17. The verification of genotypes of recombined male flies

Genotypes were tested with the primers specific to hfp, Bam-Gal4 and EP421 by PCR. Genomic DNA was isolated from single male flies. Genotypes are indicated above the images. PCR products are labeled by the sizes and names. Both Hfp alleles contain a partially excised P element in the 5′ UTR. The insertion left behind the excision was about 80 nt in hfp^{13} mutant, and 480 nt in hfp^{9} mutant. The upstream primer used for amplifying hfp alleles is located upstream of the P element insertion site and the downstream one is within the hfp intron 1 (A). The PCR product of the wild type allele is less than 300 nt. No wild type allele was detected in the hfp^{9}/hfp^{13} mutant genome (A,B). Primers internal to the Bam-Gal4 element are used to detect it, as shown in (A) no signal of Bam-Gal4 was detected in the wild type. Primers used for the EP421 element are located at the boundary of the insertion site. The element was not detected in wild type genome as shown in (A). Lanes labeled “Gal” represent the Bam-Gal4 element, “EP” represent the EP421 element.


w1118
N=10, M=75.4
% of total tested males
100
80
60
40
20
0
0
0-10
10-20
20-30
30-40
40-50
50-60
>60


Bam-Gal4/Y; hfp9/hfp13, taf1EP421 #5
N=21, M=21.19
P=0.474
% of total tested males
30
20
10
0
0
0-10
10-20
20-30
30-40
40-50
50-60
>60


Bam-Gal4/Y; hfp9/hfp13, taf1EP421 #3
N=25, M=16.76
P=0.918
30
20
10
0
0
0-10
10-20
20-30
30-40
40-50
50-60
>60


Bam-Gal4/Y; hfp9/hfp13
N=15, M=17.25
% of total tested males
30
20
10
0
0
0-10
10-20
20-30
30-40
40-50
50-60
>60

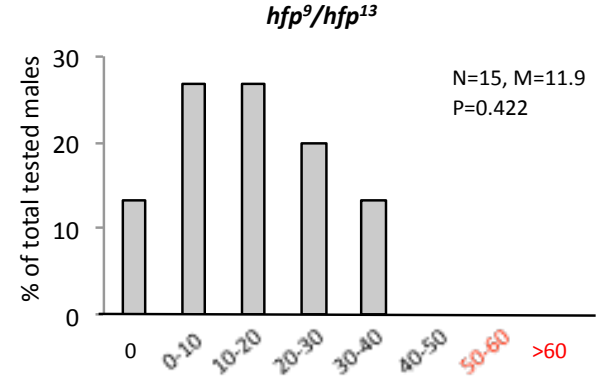

hfp9/hfp13
N=15, M=11.9
P=0.422
% of total tested males
30
20
10
0
0
0-10
10-20
20-30
30-40
40-50
50-60
>60

Figure 4-18. Fertility rescue test of Hfp mutant males by overexpressing Taf1 in testis

Individual males flies from different genotypes were crossed with wild type virgins. Percent of tested males with different numbers of offspring are summarized in the graphs. Genotypes are shown above the graphs. The numbers of tested males are indicated (N). The mean number of progeny (M) are indicated. P values of each group are compared with the control genotype *Bam-Gal4; hfp^{9}/hfp^{13}*.

Discussion

Although Hfp and Tra2 are both identified in the regulation of Taf1 exon 12a splicing, and some similar phenotypes are observed in mutants, one fundamental question is whether this alternative splicing event has any biological significance or is important for the normal spermatogenesis. As a transcriptional regulator, Taf1-2 is highly enriched in Drosophila testis. Taf1 protein has two AT hook motifs, one is encoded in exon 12 and the other one is encoded in exon 12a. So only Taf1-2 and Taf1-4 contain both AT hook motifs in their final protein products. Previous studies have shown that both AT-hook motifs are required for Taf1 to bind to DNA target efficiently. Thus alternative splicing of exon 12a is predicted to directly affect the transcriptional activity of Taf1's downstream targets, such as beta-tubulin, string and Don juan (Metcalf and Wassarman, 2006). By regulating exon 12a splicing, Hfp and Tra2 are potentially able to affect the binding activity and further the transcription function of Taf1. Therefore the regulation of exon 12a inclusion influences the process of male germ cell development. The phenotypic similarities between Hfp and Taf1 mutants shown in our experiments suggest this prediction and the importance of Taf1-2 alternative splicing during spermatogenesis. Additional experiment with Taf1-2 specifically expressed in the testis of Hfp mutant background would verify this idea. Partial rescue of the phenotypes in Hfp mutants would be expected by Taf1-2 overexpression.

We discovered in Chapter 3 that Hfp and Tra2 together repress M1 intron splicing, but here the same two proteins regulate Taf1 exon 12a splicing in opposite directions. Our in vivo experiments suggested that these factors act in in parallel pathways to affect Taf1. This conclusion is based on the observation that overexpressed Tra2 in vivo didn't result in more exon 12a skipping. Why might an increase in Tra2 affect exon 12a? Tra2 is known to be expressed at relatively high levels in the male germline (Mattox and Baker, 1991). As mentioned before, it is thought that tra2 uses a stronger promoter to reach high protein level in a short time and that negative feedback regulation is needed to limit these levels from becoming deleterious. So it is likely that Tra2 levels are not a limiting factor in promoting exon 12a skipping yet sharp reductions in Tra2, as can result in dramatic changes. So although Hfp is able to negatively regulate Tra2 through M1 splicing, this limitation in Tra2 levels has no impact on Taf1 splicing. Rather our results suggest that Hfp affects Taf1 splicing through a separate pathway.

If exon 12a splicing is oppositely regulated by Tra2 and Hfp, it might be predicted that in Tra2 mutants, the number of spermytocytes in each cyst should be more than 16 or doubled to 32. It is reported that in Drosophila ovaries (Van Buskirk and Schupbach, 2002), Encore (enc) mutants contained 32-cell egg chambers, while Hfp mutants had 8-cell egg chambers. These two genes were thought to antagonize their functions in mitosis during

oogenesis through *ovarian tumor*'s (otu) activity. Especially Hfp was shown to regulate otu splicing in this process. Therefore it was thought that Otu was responsible for the mitotic cell division in oogenesis. However Otu is not thought to express in testis, therefore we hypothesized the regulation of taf1 splicing in testis would be responsible for the cell division during spermatogenesis. However, our results from two different tra2 mutants showed only 16 spermatocyte cysts in the testes. That suggests that Tra2 probably plays limited roles on the mitotic division, or some other factors control mitosis are independent of Taf1 regulation.

Genes participating in a common regulatory pathway often share similar phenotypes. We predicted that some overlap in phenotype between Hfp and Taf1 mutants would be expected if the regulation of Taf1-2 production is dependent on Hfp. The phenotypes observed from both Hfp mutant and taf1 RNAi knockdowns share a number of similarities which support the idea that these two proteins are in a common pathway that drives meiotic events and later spermatogenesis. Another striking similarity was observed between taf1 RNAi mutant and other reported TAF mutants, such as *no hitter* (nht) and *meiosis I arrest* (mia) (Ayyar, 2003; Hiller et al., 2004). Taf1 was reported to function together with other testis specific Tafs and also co-localize with them in testis (Metcalf and Wassarman, 2007). Testes of Taf mutants consistently showed significant delays in meiosis and aberrations in spermiogenesis (Hiller et al., 2004; Hiller et al., 2001). Testes of these mutants are generally found filled with primary spermatocytes and a small

proportion of abnormal of later stages. The phenotypes we have observed in taf1 RNAi knockdowns is very similar to this suggesting that Taf1-2 could be one member of the germline TFIID complex and have an essential role on spermatogenesis.

TFIID is a transcriptional complex containing TATA-box binding protein (TBP) and multiple TBP-associating factors (TAFs) (Matangkasombut et al., 2004). In Drosophila a common set of Tafs are generally expressed (Aoyagi and Wassarman, 2001), while there is another set of Tafs that are testis specific (Hiller et al., 2004; Hiller et al., 2001; Metcalf and Wassarman, 2007). In general germ line and somatic Tafs are expressed from gene paralogs. However Taf1 is encoded by only one gene within the Drosophila genome (Wassarman et al., 2000). The expression of a germ line specific form of this protein (Taf1-2) is instead accomplished through alternative splicing. In our experiments, we showed that the testis-specific product of Taf1 is regulated by Hfp and Tra2. Exon 12a inclusion both causes Taf1-2 enrichment in testis, and also is critical for the normal development of the male germline.

The phenotypic similarities between Hfp and Taf1 mutants were also supported by testing the molecular markers involved in meiosis. Both cyclin A and twine were shown increased in Hfp and Taf1 mutant testis. Cyclin A is a cell cycle related factor that reaches its highest level in primary spermytocytes and disappears abruptly before metaphase I. Its degradation is required for spermytocytes to continue meiosis (Lin et al., 1996). In our study, cyclin A mRNA is dramatically increased in taf1 mutant testis. This could be due to the up-regulation of one or more factors that drive

cyclin A expression in the taf1 mutant background. More likely it is because more primary spermytocytes accumulated in the testis and meiosis is significantly delayed. Twine is the Drosophila homologue of cdc25 in mammals and is specifically involved in meiosis in both the male and female germ line. Its mRNA first appears in early spermytocytes and further accumulates in primary spermytocytes. It is required for the initiation of the first meiotic division in testes (White-Cooper et al., 1998). Its protein level is more critical than its mRNA level for the meiosis initiation (White-Cooper et al., 1998). In some Taf gene mutants, the testis where meiosis is blocked, Twine levels are observed to be decreased compared with that in wild type. In the Hfp and Taf1 mutant testis, the level of Twine level is not decreased which is consistent with the observation that meiosis does happen in these two mutants even though the process is impaired as shown in figure 4-14. The dj gene is required for the formation of spermatid and mature sperm and also implicated in normal Nebenkern formation (Santel et al., 1998). In vitro studies have shown that dj could be a potential transcriptional target of Taf1 (Metcalf and Wassarman, 2006). Its decrease in both of Hfp and Taf1 mutant testis is consistent with our testis staining result that abnormal Nebenkern were formed and failed elongated sperm head were seen. Also this result supports the idea that Hfp regulates spermatogenesis partially through regulating the splicing of taf1 exon12a.

Another interesting question is how Hfp can help Tra2 repress M1 splicing but promote exon 12a splicing? What differences might

exist in these substrate sequences that would be responsible for the different behaviors of the same splicing factors? Exon 12a has a weak 3′ splice site in the upstream intron. It also contains two CAAGR elements like those found in the ISS of the M1 intron as well as a CACAGG that resembles the binding site of Tra2 in dsx enhancer. These three elements are very close to the 3′ splice site upstream of exon 12a and may participate in exon skipping. Studies in mammalian cells showed that the Hfp homolog, PUF60, could recognize weak 3′ splice sites and promote RNA splicing (Page-McCaw et al., 1999; Valcarcel et al., 2007). Also evaluations of the splice site strength in Taf1 pre-mRNA showed that exon 12a contained a moderate 3′ splice site while the 3′ splice site in exon 13 was even weaker. Hfp could function in both of the 3′ splice sites and help exon 12a inclusion. However we don't have information of Hfp binding specificity, it is still not clear how Hfp can promote exon12a splicing.

Alternative splicing is a process that is extensively observed within the transcriptome. Most genes are encoded by multiple exons and their mRNAs are alternatively spliced (Wang et al., 2008). With variation in developmental stage or conditions, many alternative splicing events can be observed switched more or less in either direction. Many splicing changes have been reported in fly mutants and RNAi-treated cells. However, little is know that within these splicing changes, which one has a significant consequence and which are simply indirect noisy effect of other factors. In our study, we discovered that a small exon of Taf1 can be regulated by two

splicing factors and this splicing event probably is important for normal meiosis of germ cell development in fly testis.

Summation And Future Directions

M1 intron retention mediated by Tra2 is only seen in Drosophila male germ line. However, this regulation occurs in response to high transcriptional levels of Tra2 in the germline rather than to a tissue specific factor (Mattox and Baker, 1991; Qi et al., 2006). Therefore, although this regulation is tissue specific in vivo, many cell types retain the competence to exert M1 repression and it has been observed that M1 retention can be induced in either somatic tissues or cultured cells by elevating Tra2 level (Qi et al., 2006). We took advantage of this by using splicing reporters in a Drosophila S2 cell based RNAi screen to identify Hfp/Puf68 as a participant in the splicing repression of M1 intron both in cultured cells and in vivo. Both Tra2 and Hfp were regarded as splicing activators based on studies of other substrates (Inoue et al., 1992; Ryner and Baker, 1991; Van Buskirk and Schupbach, 2002). However they both displayed negative regulatory function on M1 intron splicing (Chandler et al., 2003; Mattox and Baker, 1991; Qi et al., 2007). More interestingly, both of these factors themselves have multiple protein isoforms but different behaviors were observed for their effects on M1 splicing (Mattox et al., 1996; Mattox et al., 1990). We found Hfp had two isoforms that differ from each other in the N terminus containing four serine-arginine dipeptides. The two isoforms were verified in vivo, and their functional differences in S2 cells may be due to the distinct patterns of subcellular localization. It will be interesting to know whether similar distribution patterns are also

present in tissues. Besides the localized distribution, a more important question for these two isoforms is whether there are any other functional differences between them. Does Hfp58 have the same effect on the regulation of c-myc transcription as Hfp68 does? Does Hfp58 also have a splicing function that is relevant in other RNA substrates? If so, does Hfp58 display a positive regulatory role in splicing? Like Tra2 that is predicted to have three kinds of protein isoforms, the functions that each isoform of Hfp takes can be tested by introducing them individually into Hfp mutant background.

Curiously, the regulators identified so far to be involved in repression of M1 intron splicing are all known to act as splicing activators in other contexts. Tra2 and Rbp1 both promote female specific splicing of both *dsx* pre-mRNA and *fruitless* pre-mRNA (Heinrichs and Baker, 1995; Heinrichs et al., 1998; Ryner and Baker, 1991). Hfp favors exon inclusion in the alternative splicing of *Otu* pre-mRNA in Drosophila oocytes (Van Buskirk and Schupbach, 2002). Yet our findings here implicate Tra2 and Hfp as co-repressors of M1 and Rbp1 was shown to be able to repress M1 splicing both in living S2 cells (Kumar and Lopez, 2005) and nuclear extracts (Qi et al., 2007) in previous studies, even though it showed minor activation activity in our reporter based screen. A challenging question for the regulation of alternative splicing therefore is how these splicing activators function together to repress RNA splicing of M1 intron? It has been shown that Tra2 could repress M1 splicing through the CAAGR repeats of ISS element

in vitro and Rbp1 functions independently through a small fragment outside of the repeats in the ISS. In our studies, we found the negative regulatory function of Hfp requires both the ISS and a weak 3′ splice site in the intron. The phenomenon of position-dependent regulation of alternative splicing has been reported in several splicing factors. Their regulatory roles on RNA splicing depend on where they bind, upstream or downstream of the regulated exons. Similarly Tra2 and other SR factors exert position dependent effects on alternative splicing as discussed in Chapter One. Upon binding to exons these factors help to promote exon inclusion but repress splicing when they are bound to introns. Notably each of the factors implicated in M1 repression, act through sequences located within the intron. However a detailed explanation of the mechanism of splicing repression by SR factors has yet to be defined. Why these factors could not just promote recognition of a weak 3′ splice site by binding to the nearby intronic elements is unclear. Exon definition by an SR protein-containing complex offers one potential explanation. Based on the result of our study we speculate that Hfp represses splicing by potentially competing with the activity of U2AF50 within the 3′ region of the M1 intron and form another more stable complex across the whole M1 intron with Tra2 that binds to the ISS. Eventually this big complex could communicate with complexes formed in the flanking exons and define the entire exon3-M1-Exon4 fragment as a single exon. This model not only can explain the phenomenon of SR-mediated splicing repression, but also is consistent with the observed differences in the effect of exon verses intron bound SR factors. Usually a weak splice site

is required for the regulation of alternative splicing and Hfp's vertebrate orthologue PUF60 has been specifically associated with recognition of weak 3′ splice sites. That could be the reason why Hfp is required for M1 retention and why a strong 3′ splice site could abolish M1 repression in vivo. Consistent with this I observed that when the dsx enhancer, which contains strong binding element for Tra2 was inserted into ftz intron containing a strong 3′ splice site, it still could not mediate intron repression even in the presence of elevated Tra2 level in S2 cells (data not shown). To further test the above model, an intron with strong 3′ splice site could be included upstream of exon 3 and an intron with strong 5′ splice site could be included downstream of exon 4. When the transcript is expressed in S2 cells, Tra2 would be predicted cause the whole exon3-M1-exon4 to be included with upstream and downstream exons in the final mRNA.

Alternative splicing of Taf1 exon 12a was found in our study to be regulated oppositely by Tra2 and Hfp in Drosophila testis. Because exon 12a encodes another AT hook of Taf1 protein, its splicing could potentially influence the transcriptional activity of Taf1 (Metcalf and Wassarman, 2006). Further spermatogenesis in both Hfp and Taf1 mutants were examined and similar effects between the two mutants were recorded. These observations suggest that the regulation of male germ cell development by Hfp is partially through the regulation of taf1 alternative splicing. From our rescue experiment, minor effect was seen by forced expression of Taf1 in the Hfp mutant background. To further confirm the

regulation of Hfp on Taf1 splicing, Taf1-2 isoform, the testis-enriched isoform with exon12a inclusion, should be specifically expressed in testis in the Hfp mutant background. This raises the question of how Hfp plays opposite roles of Tra2 on the exon 12a splicing. What elements within these introns or exons are required for Hfp activation but also suffice for Tra2 repression? At present we do not know if Hfp or Tra2 directly associates with taf1 transcript. By comparison with the situation in the M1 intron, the mechanism of exon 12a splicing regulation by both Hfp and Tra2 could provide deeper understanding of the regulatory roles of splicing factors in different contexts.

The splicing regulations of both M1 intron and Taf1 exon 12a are both involved in the male germline development. Germ cell development and differentiation is one of the most complex processes in multi-cell organisms that alternative splicing is known to play important roles in (Elliott, 2004; Walker et al., 1999). By studying splicing regulation, we can understand how tissue-specific isoforms are produced and how their alternative splicing is employed by such tissues to produce special functions, not only for normal germ cell growth and differentiation but also for the abnormal incidence of certain types of cancers.

The mechanism of splicing regulation in molecular level is not only an interesting topic but also critical to understand the pathology of certain diseases and further provide therapeutic choices in clinic. Recently a very promising treatment for spinal

muscular atrophy was developed by correcting the abnormal splicing of SMN-2 pre-mRNA with antisense oligonucleotides in mouse (Hua et al., 2010). More successful cases will be reported to cure diseases if the mechanisms of alternative splicing are better understood in the future.

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